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ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume is appearing simultaneously with Volumes IX and X. Number 1 of this volume will contain a biography of Dr. Loeb. It is to appear after Number 6, and the page numbers will be roman numerals. The publication of this volume began September 18, 1925.

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ULTRA-VIOLET ABSORPTION SPECTRA OF CERTAIN PHYSIOLOGICAL FLUIDS.

By MELVIN C. REINHARD.

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(Accepted for publication, June 3, 1927.)

The study of the absorption curves in the ultra-violet region of the spectrum has been confined chiefly to the blood serum and its proteins. Lewis[1] has examined the ultra-violet absorption of blood serum and claims to have found in this way the existence of specific changes in the blood with certain diseases. This same author[2] has also published absorption curves of the three chief proteins of the serum, namely albumin, pseudoglobulin and euglobulin. Tadokoro[3] and Nakayama[4] have observed changes in the blood serum under various conditions of physiological interest. Stenstrom and Reinhard[5] have studied the ultra-violet absorption spectra of blood sera in relation to infectious diseases and to cancer. Dhéré[6] has investigated the ultra-violet absorption of certain proteins and amino acids and Ward[7] has published the absorption curves of some amino acids.

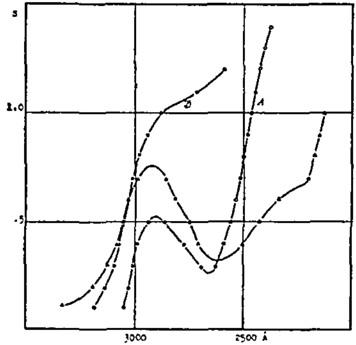
It is the purpose of this paper to present such ultra-violet absorption curves of physiological substances which would seem of value or interest to others.

Procedure.

The instrument in which the solutions were tested for their absorption in the ultra-violet was a Hilger quartz spectroscope, size *C*, with a Hilger sector photometer; the latter has two rotating sectors,

[1] Lewis, S. J., *Proc. Roy. Soc. London, Series B*, 1921–22, xciii, 178.
[2] Lewis, S. J., *Proc. Roy. Soc. London, Series B*, 1915–17, lxxxix, 327.
[3] Tadokoro, T., *J. Infect. Dis.*, 1920, xxvi, 1.
[4] Tadokoro, T., and Nakayama, Y., *J. Infect. Dis.*, 1920, xxvi, 8.
[5] Stenstrom, W., and Reinhard, M. C., *J. Cancer Research*, 1925, ix, 394.
[6] Dhéré, D., L'absorption des rayons ultra-violets, Fribourg, 1909.
[7] Ward, P. W., *Biochem. J.*, 1923, xvii, 903.

one of which, the upper, is adjustable and controls the comparison spectrum. The source of light is an under-water spark[8] which gives a continuous spectrum in the ultra-violet practically free from lines. The path of the light lies only through water, air and quartz. The range of the wave-lengths is from 7000 Å. to 2100 Å.; the width of the slit was 0.1 mm. The plates are approximately 25 by 10 cm., and can record as many as fifteen exposures; each absorption spectrum has



o Urine. △ Uric acid.

FIG. 3. Two samples of urine from the same individual, taken at different times. For Curve *A* the urine was diluted 1 part to 120 parts with water. For Curve *B* it was diluted 1 to 100. Absorption cells in both samples were 1 cm. thick. A comparison of Curve *A* with that of uric acid shows a marked presence of uric acid in this sample. The uric acid curve was obtained by using a M/1000 solution of uric acid in water with a 0.1 cm. cell.

contiguous to it a comparison spectrum by means of which the points of equal blackening are found; a wave-length scale is photographed at the top and at the bottom of the plate; its correct placement is determined with the help of the lines from copper.

[8] Set up as described in *Sc. Papers Bur. Standards*, 1922, xviii, 128, except that the two rotating zinc discs for the outside spark are replaced by two adjustable horizontal zinc rods, with a blast of air directed into the gap.

covering the slit. The fixed sector is fitted with a cell, with quartz windows, which is filled with the solution to be tested.

Once the proper dilution has been determined by preliminary tests, the cell is filled and no further change is necessary; instead of further dilution, or change in the thickness of the cell, the period of illumination is varied; the short exposure corresponds to low dilution, the long exposure to high dilution. The exposures which have a value

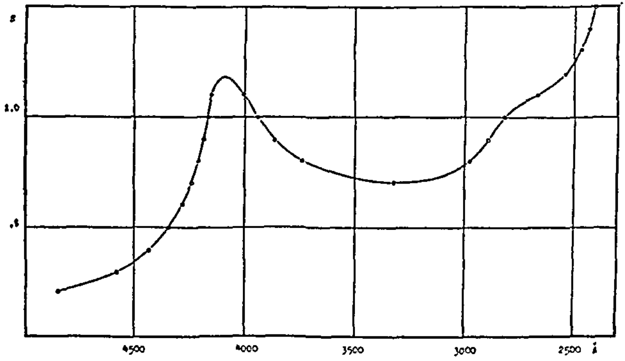


FIG. 5. For the curve of hemoglobin, 30 mg. of Pfanstiehl hemoglobin were dissolved in 250 cc. of water. Thickness of cell, 5.0 cm.

lie between two limits: the shortest exposure must cause on the plate no measurable blackening, the longest exposure must show almost continuous blackening and, hence, must no longer show one or more bands; the exposures showing the location of the band lie between these two limits.

The points at which the blackening in the absorption spectrum is equal to the blackening of the contiguous comparison spectrum are determined, in the dark room, by placing the plate on a ground glass illuminated from beneath; the approximate location is noted, then all but a small area surrounding the point is covered by a sheet of black paper fitted with an elliptical hole; with the aid of a lens mounted

ORIENTATION IN COMPOUND FIELDS OF EXCITATION; PHOTIC ADAPTATION IN PHOTOTROPISM.

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(Accepted for publication, June 18, 1927.)

I.

One test of tropistic analysis is its usefulness as providing a description of oriented movement under controlled circumstances of deliberately contrived simplicity. A deeper test is found in its ability to predict measurable aspects of behavior in new situations of some complexity. It should be more obvious than it seems to have been, that an understanding of certain features of animal movements may be arrived at through the attempt to synthesize reasonably complex situations of which significant elements may be studied separately. It is in fact frequently easier to measure the effect resulting from controllable opposed sources of excitation than to quantitate single orienting tendencies. Experiments of this type should therefore give, in suitable instances, a convenient method for the precise characterization of orienting effects (Crozier, 1924–25, 1925–27; Crozier and Stier, 1927–28). They may also open a way toward the measurement of certain aspects of central nervous function (*cf.* Crozier, 1925–27; Crozier and Pincus, 1926, 1926–27, *a*) and of important features of quantitative variability in behavior (Crozier and Federighi, 1924–25, *a;* Crozier, 1925–27).

We have been interested to secure data upon additional instances to supplement the now scanty information bearing upon such questions. The treatment given by Szymanski (1911, 1911–12) does not meet our requirements; nor, for other reasons, does the interpretation of Weiss (1924), so far as we are able to understand it. With an organism which is both geotropically reactive and phototropic, a compound field of excitation may be devised such that in creeping the organism

* Fellow of the International Education Board.

is forced to resolve a conflict between mutually opposed orienting tendencies. If, from previous knowledge of each tropism as a more or less isolated element, it be possible to predict the form taken by the resolution, then there is obtained good indication of the efficacy of the initial interpretations. For the phototropic orientation of young rats upon an inclined plane it was possible to predict that the intensity of the light just required to enforce horizontal creeping should be a power function of the sine of the inclination of the creeping plane (Crozier and Pincus, 1926–27, *c*); and it was possible, also, to account for the peculiar changes in the variability among the individual measurements at successively greater inclinations. Until it has been possible to carry analysis of this type into the treatment of cases still more complex, it would appear to be a waste of time to speculate extensively, as so many have done, regarding imponderable elements supposed to characterize behavior.

II.

The geotropic creeping of the slugs *Limax maximus* (Crozier and Pilz, 1923–24; Crozier and Federighi, 1924–25, *c*; *cf.* also Crozier and Pincus, 1926–27, *a*) and *Agriolimax campestris* has been carefully examined, particularly in the case of the latter species (Wolf, 1926–27). On a plane inclined at angle α to the horizontal the slug's orientation (upward or downward) is limited by an angle θ on the plane, such that, very nearly

$$\frac{\Delta\,\theta}{\Delta\,\log\,\sin\,\alpha} = const.;$$

and, better,

$$(\Delta\,\sin\,\alpha\,)\;(\Delta\,\sin\,\theta) = const.$$

The phototropism of these slugs has also been studied extensively, especially in the case of *Limax* (Crozier and Federighi, 1924–25, *b*; Crozier and Libby, 1924–25; Crozier, 1925–27); data from experiments of Crozier and Cole relative to circus movements (*cf.* Crozier and Cole, 1922) as a function of light intensity are as yet unpublished, but a brief account has been given of orientation with opposed sources of light (Crozier, 1925–27). *Agriolimax* differs from *Limax* notably in the greater speed with which it becomes adapted to light

of ordinary intensities, as we shall describe. This in fact makes it possible to study here the kinetics of light adaptation. For *Limax* it was found that the amplitude of turning in circus movements, with constant speed of creeping, is very nearly proportional to the logarithm of the light intensity (Crozier and Cole). (This of course need not mean that the primary effect of the light follows this law, since the amplitude of turning is related to the speed of linear progression.)

Experiments were devised to examine the behavior of *Agriolimax* in a compound field of excitation such that the animal should at the same time be caused to orient upward upon a vertical glass plate, and laterally by a beam of light. The slug being initially dark-adapted, time enters as a variable under continuous exposure since photic adaptation is fast; the enforced deviation from vertical creeping therefore decreases. The operative problem thus consists in obtaining a measure of the orienting effect of the light, with the gravitational influence constant, at brief intervals during exposure to light until adaptation is effectively complete. While it would in some ways be preferable to measure at each chosen moment the intensity of light necessary to produce a certain constant deviation of the creeping path from the vertical, we have found it much easier in the present experiments to measure the deviation itself as a function of time. It is possible to account for the course of the observations quantitatively and very simply. The procedure may be reversed in a very obvious way for the study of dark adaptation.

In this manner it becomes possible to investigate the kinetics of photic adaptation in a phototropic animal.[1] The point is an important one, because few direct studies of light adaptation have been made. The present theory of the situation is based upon Hecht's general analysis of photic irritability, and, specifically with regard to light adaptation, upon his experiments with *Ciona* (Hecht, 1918–19). In this case it was shown that the decay of an initial mass of photosensitive substance, during repetitive stimulation by light, follows the law of a first order process. The further analysis of dark adaptation and of sensory equilibrium and intensity discrimination, in *Mya*

[1] A slightly different method has been suggested in connection with circus movements (Crozier and Pincus, 1926–27, *b*).

and in the human retina, is beautifully consistent with this (Hecht, 1922–23, 1924–25, 1926–27). Hecht (1919–20, *b*) has pointed out that in such forms as *Ciona*, *Mya*, and others in which the time limit for the delivery of photic energy adequate for excitation is brief,—that is, exhibiting differential sensitivity,—the time limit is short because necessarily a function of the relative velocity of the "dark" reaction which regenerates photosensitive substance. This makes it possible to regard such cases as involving a mechanism which is fundamentally the same even when the excitation is continuous, as in phototropism. It is clearly important to determine if, in fact, similar mechanisms do underlie photic excitation in the two classes of reactions. When adaptation occurs very slowly it is almost impossible to devise the requisite experiments.

III.

A study of light adaptation here described was made with the slug *Agriolimax campestris*, already used for the investigation of geotropic conduct, so that the behavior in creeping on an inclined plane in darkness was well known. *Agriolimax* becomes adapted to light in a few minutes, so that the process of adaptation can easily be measured. The results obtained have to be carefully handled, however, because the animals are easily influenced in their response to gravity and light by food, for example, as shown by Crozier and Libby in *Limax* (Crozier and Libby, 1924–25). Furthermore, change of weather may influence the responses very strongly. The animals, even when kept for days and weeks under almost constant temperature and humidity, in a dark room, very often give no response whatever. The slugs used for the experiments were kept in darkness for at least 12 to 24 hours so that they were always well dark-adapted before the tests were made. They were then tested one after another. About 75 to 80 per cent showed a good negative orientation to gravity and to light. As pointed out before (Wolf, 1926–27), sometimes the slugs are positively geotropic. On the other hand, differences in the sensitivity to light can be observed. The animals which gave good responses were tested repeatedly. After being confined in the laboratory for 5 to 6 days, creeping and the tropistic responses may become too slow to permit proper determination of angles of orienta-

tion. During an experiment one animal was put upon a vertical plate of ground glass which was moistened every few minutes. In darkness the animals typically then creep straight upward. At one side of the creeping surface is a source of light which gives an equal distribution of parallel horizontal rays. Under these circumstances the animal moves upward, and at the same time away from the light with a certain angle of orientation (θ) (Fig. 1). After an interval of 1 minute the animal is taken away from the glass plate and put back, with vertical orientation, at the same place where it started its

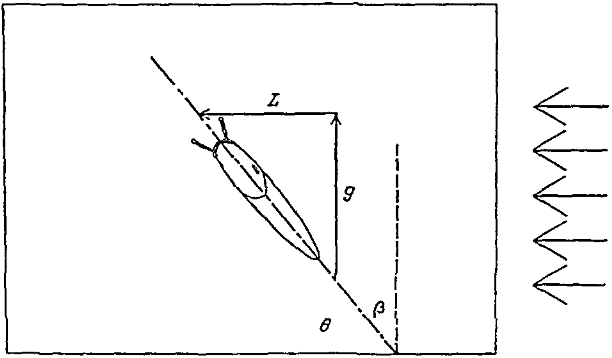

FIG. 1. Orientation of negatively geotropic and negatively phototropic *Agriolimax* on a vertical plate with light from the right,—to indicate terminology of the phototropic (L) and geotropic (g) vectors, the angle of orientation (θ), and its compliment (β).

first run. The glass plate is divided in squares, so that the path of the slug can easily be copied on coordinate paper, where the angles of orientation are subsequently measured. The angle of deflection from the vertical decreases quickly. During tests with one animal the slug is repeatedly put back at the starting point, until finally the angle of deflection from the vertical becomes a minimum, sensibly zero. It is assumed that light adaptation is reached as soon as no deviation is apparent from the perpendicular path which ought to be

taken on a vertical plate in darkness; this means, of course, light adaptation to a degree which allows excitation to fall below the threshold required for effective opposition to the orienting force of gravity, as brought out subsequently. While it may appear that this method is crude, since it involves handling, it was always carried out with care and in a uniform way; the handling in fact assists in obtaining continuous creeping. The time lost during the manipulation in bringing the slug back to the starting point is about 5 to 7 seconds until it creeps again and shows a definite response. If handled carefully the eyestalks are not retracted during this operation, so that the eyes are continuously exposed to the light.

The progress of dark adaptation can also be observed in this way. For such studies the animals are first exposed to light until complete light adaptation is reached. The methods used during the experiments are the same as with light adaptation, but the slug is run at intervals after being placed in darkness. Dark adaptation is very much slower than light adaptation, and therefore does not interfere with the direct study of the light adaptation. In a later paper there will be given a fuller treatment of the results obtained during these observations. When tested with light of about 48 f. c. intensity, dark adaptation is not complete until approximately 4 hours.

It is to be noted that in these experiments the light acted always upon the *right* side of the slugs. Measurements of phototropism have shown that fluctuations do occur in the sensitivity of each eye; but the photic reactivity of the two sides, contrary to some earlier statements in the literature, is essentially the same (*cf.* Crozier and Cole, 1921–22).

The first experiments were made at three different times during winter and spring, with one constant light intensity of 29.48 f. c. Throughout these experiments the light intensities were measured by direct photometry, not computed. The initial series of experiments was made with individuals collected in the middle of October at various places. The time during which the animals crept away from the light, before they were brought back to the original starting point near the source of light, was 2 minutes. Each animal was tested at intervals of 1 or 2 days, between five and ten times in all. The results obtained from these more or less preliminary experiments showed

clearly the decrease of the angle of orientation. No great value can be given to these results, because at first the population used was not homogeneous enough; the temperature in the dark room was rather low and inconstant; and the interval of 2 minutes between the successive runs was too great, since the animals moved too far away from the light and decrease of its intensity was perhaps too great. The

TABLE I.

Mean angles of orientation (θ, β, as in Fig. 1) upon a vertical plate with light from one side, with their probable errors and measures of variability. Seven animals tested five to ten times each, at intervals of 2 minutes (see text). For convenience in further treatment the angle β, compliment of θ, is also tabulated.

Series	Light intensity	Time	θ	P.E.$_m$	C.V.	β
		min.			*per cent*	
I	29.48 f.c.	1.0	36.9°	±0 65	12.6	53.1°
		3.0	53.9°	±1.2	15.9	36.1°
		5.0	67.4°	±0.89	9.4	22.6°
		7.0	75.4°	±0.68	5.9	14.6°

TABLE II.

Mean angles of orientation (θ) of *Agriolimax* upon a vertical plate with light at one side, with P.E.$_m$ and measure of variability. Four individuals, tested six times each.

Series	Light intensity	Time	θ	P.E.$_m$	C.V.	β
		min.			*per cent*	
II	29.48 f.c.	0 5	17.9°	±0.26	9.6	72.1°
		1.5	38.7°	±0.49	8.3	51.3°
		2.5	60.6°	±0.98	10.7	29.4°
		3.5	74.5°	±0.39	3.4	15.5°

measurements are summarized in Table I. The data show (Fig. 2) the gradual approach to a condition of adaptation in which the light has no effect on the orientation. As already pointed out, if the inclination of the creeping plane be quickly decreased orientation away from the light is greater. And for some time after the slug is found to creep vertically as in darkness it will still be oriented by the light if the creeping surface is lowered to a horizontal position.

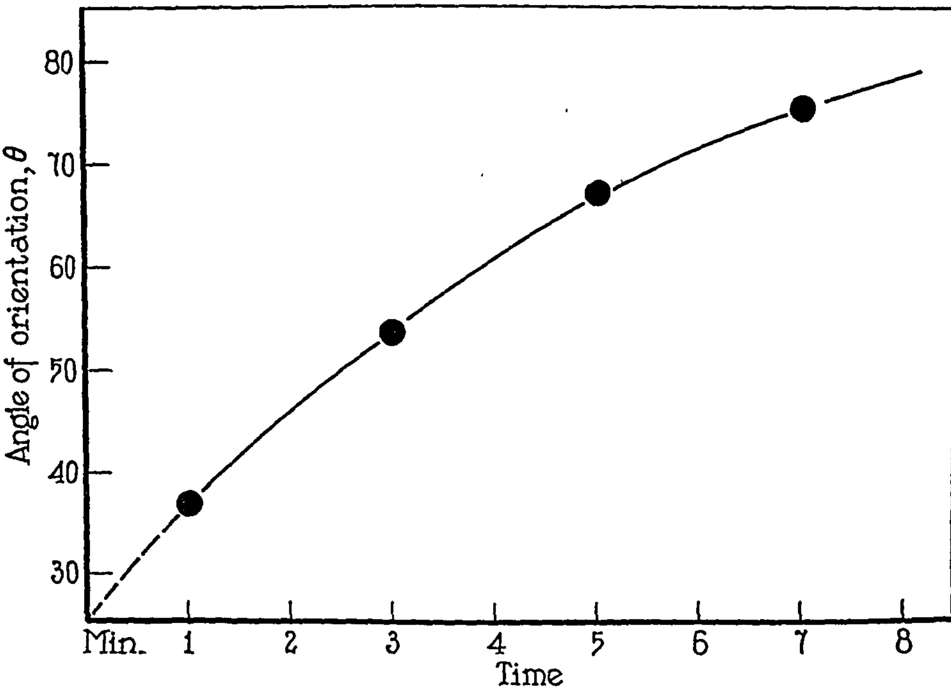

FIG. 2. The decrease of the angle of orientation (θ) with time during continuous exposure to light of 29.48 f.c. The time measurements are the mid-points of successive trails, in this case obtained at intervals of 2 minutes (Table I).

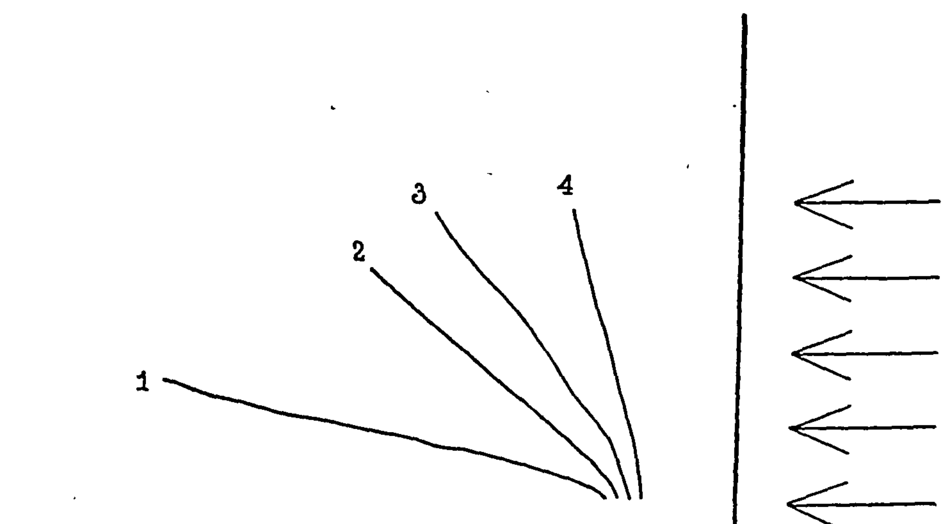

FIG. 3. Successive trials made by initially dark-adapted *Agriolimax* on a vertical plate, light from the right, at intervals of 1 minute (mid-points). (This gives the form of the records as available for analysis; actually the trails all began at the same point.)

For all later tests animals were collected at one place in the greenhouses of the Bussey Institution, which promised to give a more uniform population. The interval between the successive runs with each animal was only 1 minute. The path made during this time by the slug away from the light becomes therefore so short that the decrease of light intensity and the changing state of adaptation stay within limits which can be more easily neglected (Fig. 3).

The second series of experiments was made at the end of January and the beginning of February. For this series only six animals were used, which were tested six times each. Table II gives the measurements of the angles with the means and their probable errors. The light intensity was the same as used in the first series of tests.

TABLE III.

Mean angles of orientation (θ) of *Agriolimax* upon a vertical plate with light at one side, with P.E.$_m$ and measures of variability. Four animals tested six times each.

Series	Light intensity	Time	θ	P.E.$_m$	C.V.	β
		min.			*per cent*	
III	29.48 f.c.	0.5	27.9°	±0.69	17.4	62.1°
		1.5	46.7°	±0.48	7.2	43.3°
		2.5	60.6°	±0.34	3.7	29.4°
		3.5	72.6°	±0.41	3.7	17.4°
		4.5	79.0°	—	—	11.0°

The third series was carried on at the end of March; four animals were each tested six times. The results are summarized in Table III. They can properly be compared with those obtained 1 month before; even quantitatively the results are closely similar.

IV.

When a slug is creeping on a vertical plane illuminated from one side, two forces act upon it. The light tends to cause photonegative orientation, and the geotropic stimulus forces the animal to creep upward. If during such a test the light be removed, orientation is immediately upward. If, on the other hand, the plate be lowered, so as to be inclined to the horizontal, orientation is more completely

away from the light. Therefore the actual outcome depends upon concurrent continuous stimulations of the two kinds. The path actually described may then be regarded as the resultant of these two components. The deflection from vertical (β) is measured. Referring to Fig. 1, over a short interval

$$\tan \beta = \frac{L}{g}, \tag{1}$$

where L signifies the orienting effect of the light and g represents the geotropic response.

In describing geotropic orientation it has been pointed out that on a plane inclined at angle α to the horizontal upward movement is limited by an angle θ on the plane, such that

$$\frac{\Delta \theta}{\Delta \log \sin \alpha} = const.$$

(Crozier and Pincus, 1926, 1926–27, *a*, *d;* Wolf, 1926–27). But the attainment of such orientation does not mean that geotropic excitation ceases. The expressions relating to the angle θ merely define the extent of orientation. When orientation is achieved, the rate of progression (*cf.* Crozier and Pincus, 1926–27, *a;* Cole, 1925–27; Pincus, 1926–27) remains a function of the active gravitational component. This is true of *Agriolimax* and certain other forms, as well as of those cited, although extensive measurements have been made only with rats (Pincus, 1926–27). In this case, the rate of oriented creeping is also directly proportional to $\log \sin \alpha$. We may assume that this is likewise true for the slugs. It means that, θ and the rate of linear progression being each proportional to $\log \sin \alpha$, the rate at which the organism tends to lift its mass vertically upward is obviously proportional to $\log \sin \alpha$. From this it is fair to assume that the intensity of geotropic excitation is constant, for each value of α, regardless of the position of the animal's axis on the creeping plane—although the extent of upward orientation is limited by the distribution of tensions on its peripheral musculature.

If we then take g in equation (1) as constant, since the present experiments involve only $\alpha = 90°$, we have

$$k \tan \beta = L. \tag{2}$$

But L changes with time, due to light adaptation. We assume that at any moment the photic irritability is proportional to the amount of photosensitive material S, only very slowly formed by the proc-

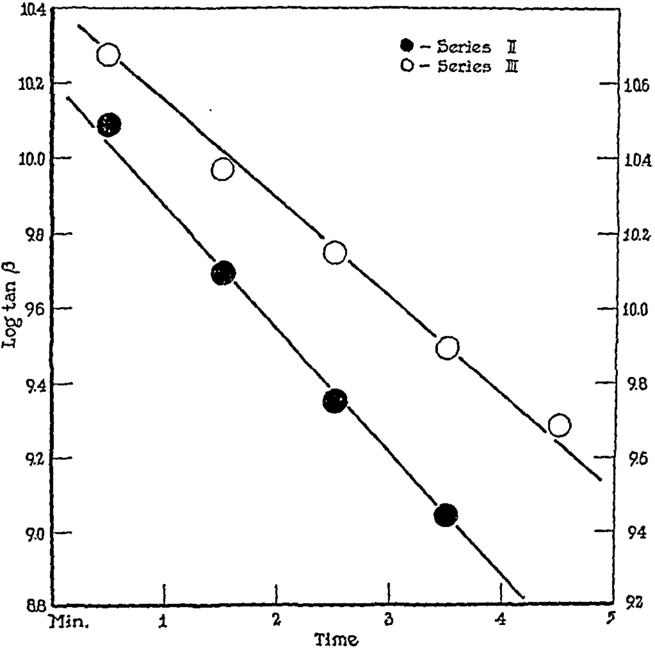

FIG. 4. The rate of light adaptation in two series of experiments made at different times (see text). Series II, scale at right. Series III, scale at left; intensity = 29.48 f.c. The results are plotted in terms of the equation deduced in the text, and if it be applicable they should give a linear decrease with time.

esses underlying dark adaptation, and that the photochemical destruction of S follows the kinetics of a first order reaction (*cf.* Hecht, 1922–23, 1923–24, 1924–25). This means, simply, that the freshly formed

decomposition products of S, requisite for excitation, will ove short interval be proportional to

$$S_t = S_0 e^{-K't}.$$

Since the light intensity is constant during an experiment we write

$$K'' \tan \beta = e^{-K't},$$

where K'' is a proportionality constant, K' a velocity con From this,

$$\log K'' + \log \tan \beta = - K'''t,$$

where K''' includes the modulus.

The constant K''' may be determined graphically; K'' includ many proportionality factors that its discussion is postponed experiments have been completed at various angles of inclinatio of the creeping surface. The formula states that the logarith the tangent of the angle should decrease linearly with time d light adaptation. The results already given in Tables I to and subsequently, show excellent agreement with this expecta (Fig. 4).

V.

If the variation of the angle β with time during light adapta may thus be used to follow the kinetics of the process, it is t expected that K''', the "velocity constant," should be a functio the light intensity—and presumably, over ordinary ranges of tensity, a logarithmic function (*cf.* Hecht, 1919–20, *b*, 1920– Experiments were therefore made with intensities, 73.69 and 1 f.c., respectively above and below the value initially used. results are given in Table IV. It is to be noted that the data closely comparable whether the averages are based upon a l number of individuals tested only once or twice, or upon a few mals tested repeatedly.

In Fig. 5 the data of Table IV are plotted in terms of equation The agreement may be taken to be very good. The slopes of lines in Figs. 4 and 5, given in Table V, change with the intensity

TABLE IV.

Mean angles of orientation (θ) of *Agriolimax* upon a vertical plate with light at one side, with P.E.$_m$ and measures of variability.

Series	Light intensity	Time	θ	P.E.$_m$	C.V.	β
17 animals tested once each.						
		min.			*per cent*	
IV	16.32 f.c.	0.5	58.0°	±1.18	12.3	32.0°
		1.5	71.4°	±0.75	6.3	18.6°
		2.5	79.5°	±0.61	4.5	10.5°
		3.5	84.0°	±0.75	1.8	6.0°
16 animals tested once each; same as used in Series IV.						
V	16.32	0.5	60.5°	±0.62	5.9	29.5°
		1.5	70.1°	±0.71	5.5	18.9°
		2.5	78.6°	±0.40	2.9	11.4°
6 animals tested six times each.						
VI	73.69	0.5	30.8°	±0.30	7.9	59.2°
		1.5	53.8°	±0.67	9.9	36.2°
		2.5	71.4°	±1.15	12.8	18.6°
		3.5	81.0°	±0.32	4.8	9.0°
25 animals tested once each.						
VII	73.69	0.5	22.5°	±1.35	44.4	67.5°
		1.5	48.3°	±1.49	21.9	41.7°
		2.5	67.8°	±1.10	11.8	22.2°
		3.5	78.7°	±0.65	4.1	11.3°
23 animals tested once each; same as used in Series VII.						
VIII	73.69	0.5	21.3°	±1.27	44.8	68.7°
		1.5	49.0°	±0.91	12.4	41.0°
		2.5	68.4°	±0.94	9.2	21.6°
		3.5	80.0°	±0.81	5.4	10.0°

TABLE V.

Values of the velocity constant for light adaptation (K''' of equation (4)), as related to intensity.

Intensity	K''' Mean
16.32 f.c.	2.6
29.48	2.92
73.69	3.53

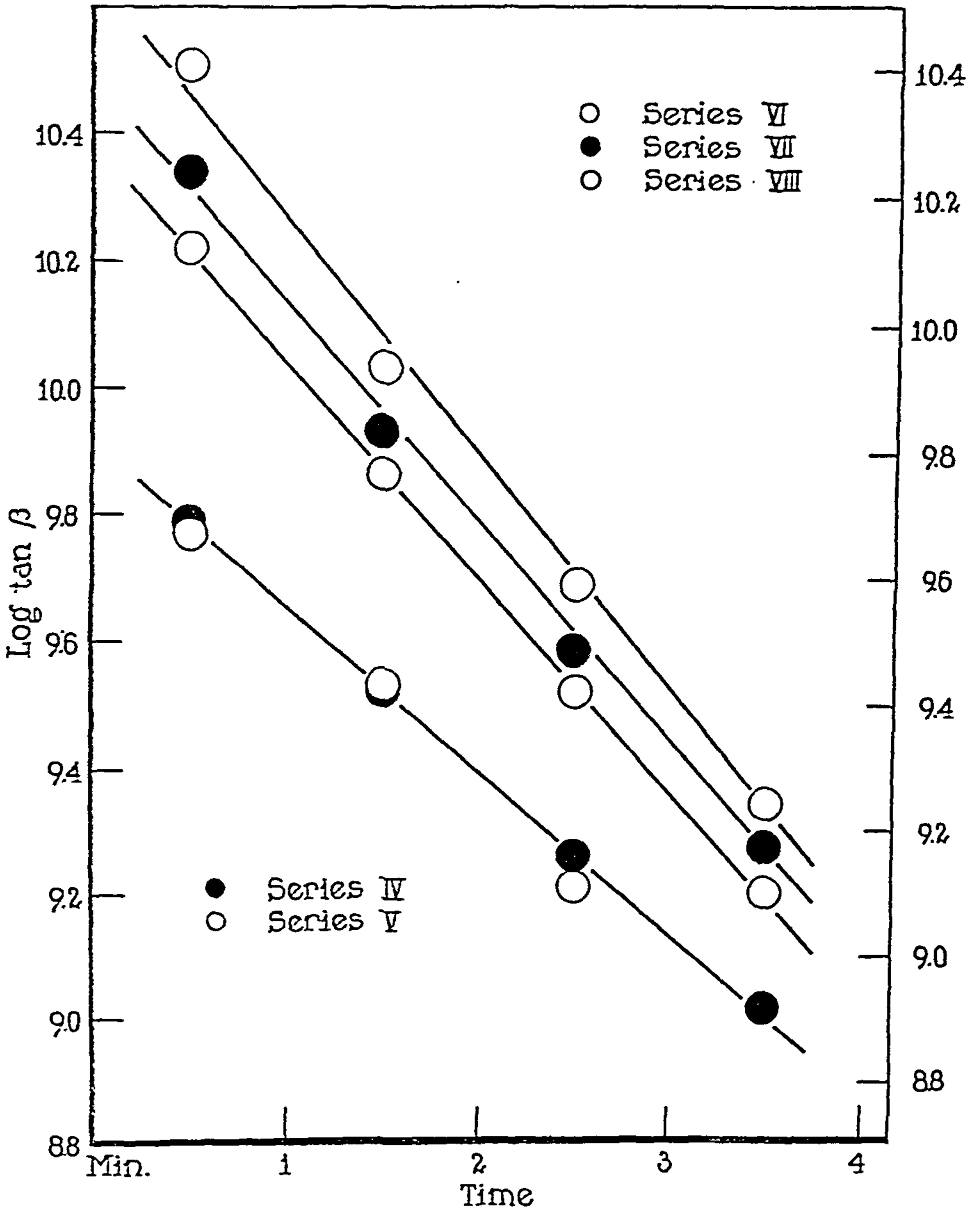

FIG. 5. The course of light adaptation in five series of experiments, at two different intensities: for Series IV, V, 16.32 f.c.; for Series VI to VIII, 73.69 f.c.

of the adapting light in such a way that K''' is directly proportional to the logarithm of the light intensity, as is shown in Fig. 6. The rate of light adaptation with intensities higher than 100 f.c. is so great as not to be measurable by this method; adaptation is complete in less than 2 minutes.

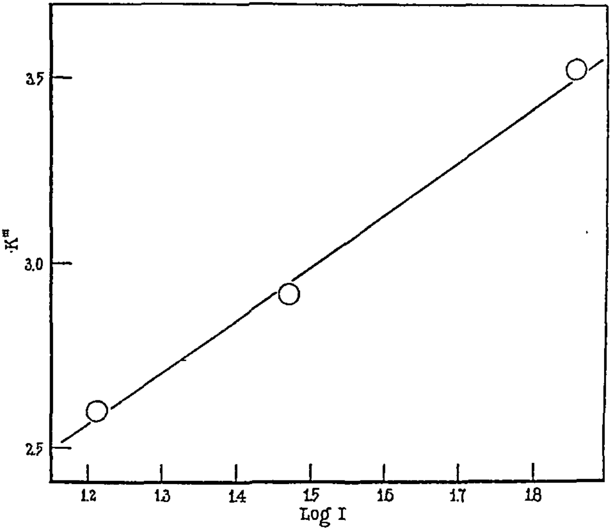

FIG. 6. The *rate* of light adaptation of *Agriolimax*, as measured through the opposition of photic and constant geotropic orienting forces, increases directly as the logarithm of the light intensity.

VI.

If a dark-adapted slug be placed at a point A, Fig. 7, on a vertical plane, with a source of parallel light rays at the right, its complete path, uninterrupted until light adaptation is attained, should be a curve more or less of the type shown. It is of interest to analyse such paths, briefly: because they give useful clues for the extension

of tropistic analysis to the consideration of non-rectilinear orientations. As such a slug creeps away from the light its own adaptation and the decreasing light intensity lead to creeping which is more and more nearly straight upward. With the experimental conditions so

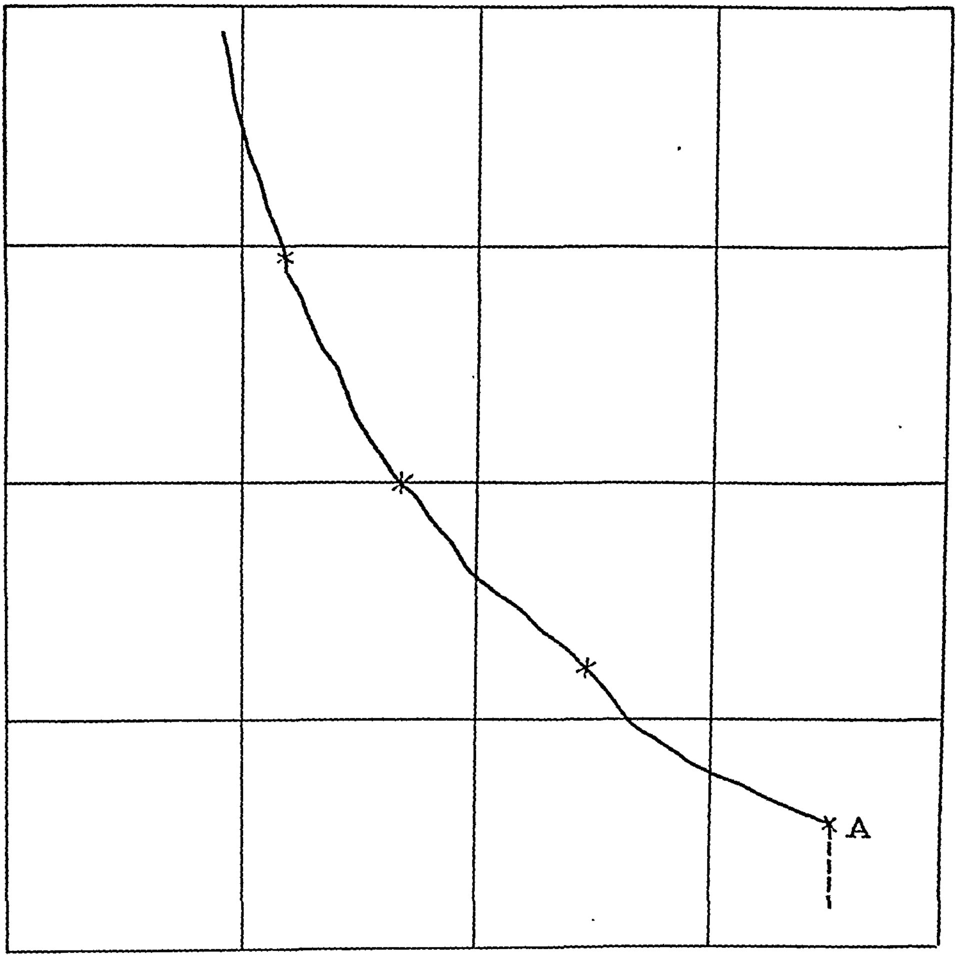

FIG. 7. Copy of the trail of a dark-adapted *Agriolimax* creeping on a vertical plate, at first in darkness (dashed line); light from the right is admitted at *A*. Successive minutes are marked on the trail. The coordinate units = 5 cm. (see text).

arranged that the light intensity does not decrease as the slug moves to the left, the slope of the curve at any point should be given by equation (3). On the other hand, if the light comes from a point

source, so that the intensity varies, the velocity "constant" for adaptation must include the logarithm of the intensity as a variable.

This could be tested by means of trails executed at different constant speeds of progression, but it is doubtful if conditions governing adaptation within the sense organ would be sufficiently uniform from time to time to permit more than a qualitative test.

SUMMARY.

During upward geotropic orientation upon a vertical plate the slug *Agriolimax* creeps vertically, in darkness. Horizontal light from one side produces orientation of dark-adapted slugs away from the vertical path, through an angle (β). The magnitude of this angle is a function of the light intensity and of time. The moderately rapid course of light adaptation is followed by measurements of β at fixed intervals. Simple assumptions as to the nature of the orienting forces lead to the conclusion that the logarithm of the tangent of β should decrease linearly with time, and that the rate of the decrease should vary directly with the logarithm of the light intensity. Both expectations are adequately realized. Certain implications of these results for behavior analysis are pointed out.

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THE RATE OF KILLING OF CLADOCERANS AT HIGHER TEMPERATURES.

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I.

At temperatures definitely supranormal duration of life becomes very rapidly less as the temperature is raised. The high values of the temperature coefficients obtained for such phenomena[1] have been compared with those found for "thermal destruction" of organic compounds and for the heat denaturing and coagulation of proteins.[2] These comparisons do not especially explain why the temperature coefficients should be so large. Nor have the majority of the published experiments considered very closely the precise form of the curve for rate of killing at higher temperatures, and the way in which this curve makes transition to that for duration of life at inframaximal temperatures. Information of this sort was desired for the location of "upper critical temperatures" for diverse forms of Cladocera, thus *facilitating their physiological characterization.* Such "upper critical temperatures" were expected to give material for comparison with critical temperatures (Crozier, 1924; 1925–26) occurring within the zone of completely reversible thermal effects. The data were obtained by determining death curves for cladocerans, clonally uniform, at each of a number of "lethal temperatures." The results permit fairly exact comparisons of the "velocities of killing," and the calculation of temperature characteristics (μ) for certain phases of this

[1] Loeb (1908); Moore (1910); Lepeschkin (1910; 1923); Goodspeed (1911); Ayres (1916); Groves (1917); Bigelow (1921); Smith (1923); Heilbrunn (1924, *a*); Klopp (1924–25); Collander (1924); Crozier and Stier (1924–25, p. 440).

[2] Arrhenius (1907; 1913); Chick and Martin (1910; 1912, *a*, *b*); Hartridge (1912); Hecht (1918–19); Lewis (1926, *a*, *b*, *c*).

process which may be compared with the results of some earlier experiments.

II.

Two species of Cladocera were used. *Daphnia pulex* was selected from a small rain-filled pond near Cambridge, Massachusetts. Large parthenogenetic females were the only kind present at that time in the pond. The animals were allowed to remain at laboratory temperature for 1 to 3 days before they were employed in the experiments. *Moina macrocopa* was secured from a clone originating from material supplied by Dr. A. M. Banta. It occurs near Cold Spring Harbor, Long Island, during the summer. The animals used were derived from one parthenogenetic female and were raised in mass cultures. Only adult females were used.

Temperature control was obtained by a water bath heated by an immersion heater with relay and mercury thermoregulator. Aggitation of the bath was by a motor-driven stirring rod. The temperature in the bath was constant to ±0.05°C. during the course of an experiment. A series of test-tubes, each containing 40 cc. of culture water, was suspended in the bath and allowed to become adjusted to the temperature of the bath. A dense culture of animals of the desired age was previously prepared so that from 15 to more than 100 animals could be drawn up into a pipette with about 2 cc. of culture water. Such a group was introduced into the first tube, then after a proper interval a similar group was introduced into the second tube, and so on until each of the tubes in the series contained animals. A proper interval was allowed to elapse after the introduction of the last group, and then the rack containing all the tubes was quickly lowered into a large jar containing cold water. The temperature inside the experimental tubes fell within the first few seconds below the temperature lethal for that species of cladoceran. The culture water and animals were then transferred to a small wide mouthed bottle and allowed to stand until the animals showed a sharp division between those dead and those alive. For *D. pulex* this time is about 12 hours, but for *M. macrocopa* 4 or 5 hours sufficed for a sharp separation. A greater delay before reexamining the animals was avoided because some of the dead animals might start to disintegrate. The individuals were tabulated as "dead" and "alive," and as many experiments were made at a given temperature and time as would provide a total of from 60 to 200 animals upon which to base one point on the mortality curve.

Experiments at different temperatures were run on the same day, so that unknown but possible irregularities in the stock would be discounted. Also, at the higher temperatures, when the time was short (5 to 50 seconds), the initial temperature in the tubes was slightly above the point desired, so that the addition of

the 2 cc. of culture water along with the animals served to bring the temperature to the right point.

In Fig. 1 the plotted points represent, for each of the several temperatures, the percentage of *D. pulex* that died after the given number of second's exposure. The temperature range is from 32° to 37°C. The curves fitted to the plotted points represent the distribution of resistances at each temperature. For 34° three points for 100 per cent dead, at 3, 4 and 5 minutes, are not shown for the sake of simplicity. One point, 36 per cent for 34°, is based on but 25 individuals; another, of 56 per cent for 32°, is based on but 16 animals. These points consequently are not given much weight. Fig. 2 shows similar curves for *M. macrocopa*. In this species the lethal temperatures are higher, ranging from 40° to 47°C. This form is more easily handled than the other and consequently the upper and lower temperature limits could be approached more exactly. No attempt was made to determine accurately the shape of the ends of the curves, but attention was chiefly directed to that portion representing from 20 to 80 per cent dead. These methods therefore differ considerably from those used by von Transehe (1913), and seem to have avoided certain sources of irregularity.

III.

Results gotten by this procedure are given in Fig. 1 (*D. pulex*), and in Fig. 2 (*M. macrocopa*). From the best fitting mortality curves, which were first adjusted in final form before any use was made of them, it was possible by interpolation to obtain the time at each temperature required to give a certain percentage of survivors. In this way figures are secured which permit comparison of the rates of the processes underlying killing at the different temperatures. Since full time was allowed for recovery, the figures used represent irreversible destruction. We have chosen to compare the times required to produce death (*a*) in 40 per cent, and (*b*) in 70 per cent of the individuals. The reciprocals of these times are plotted logarithmically, in Figs. 3 and 4, against $1/T°$ abs. The graphs are almost or quite rectilinear between 32° and 37° for *D. pulex*, and between 42° and 46° for *M. macrocopa*. The critical thermal increments for 40 per cent and for 70 per cent killing are sensibly identical for *D. pulex*, being $\mu = 119{,}400$. The values of μ for *M. macrocopa* are not quite the same at 40 per cent and at 70 per cent, but the difference is in all likelihood within the probable

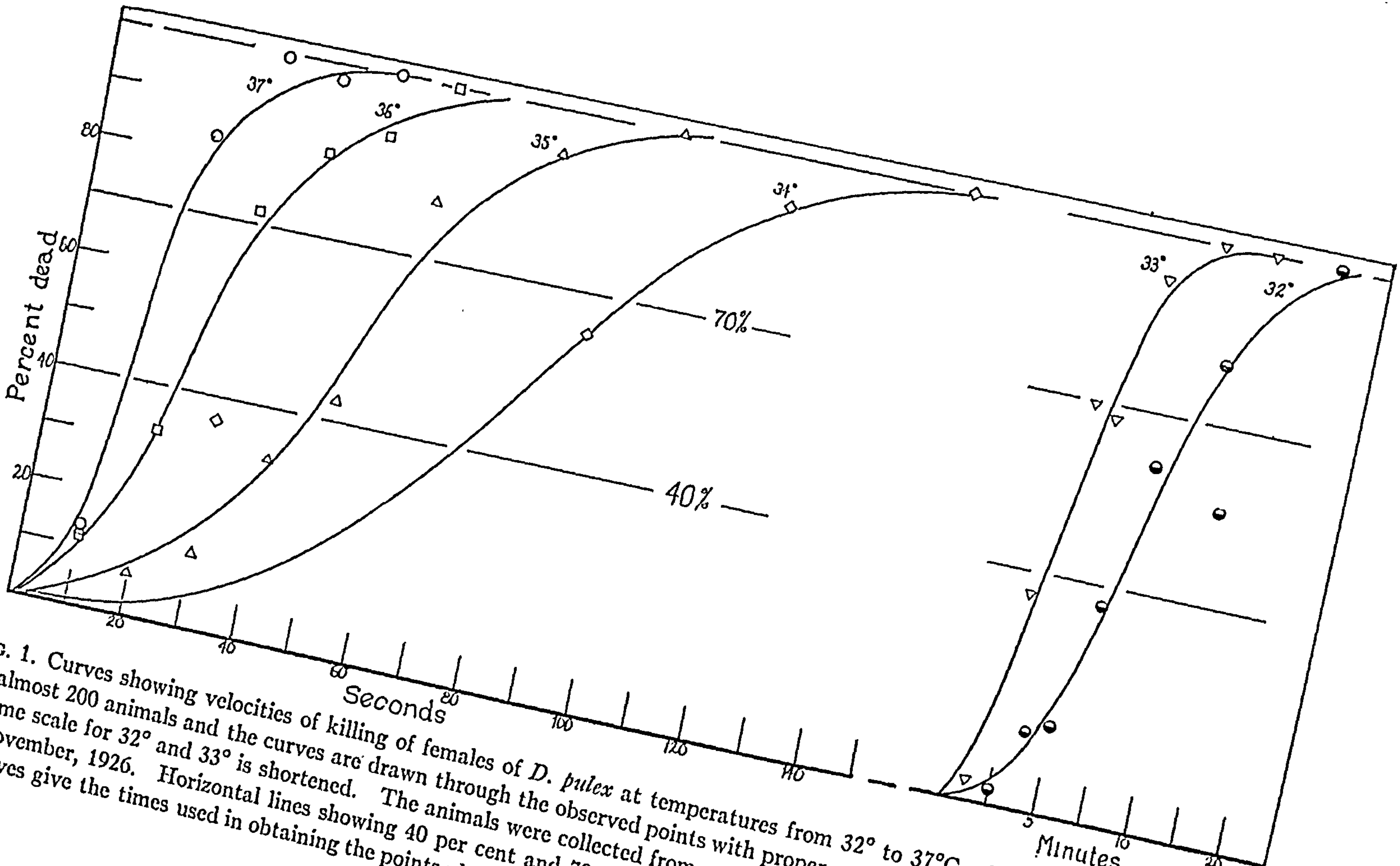

FIG. 1. Curves showing velocities of killing of females of *D. pulex* at temperatures from 32° to 37°C. Each point is based on from 25 to almost 200 animals and the curves are drawn through the observed points with proper regard to the relative weights of the points. The time scale for 32° and 33° is shortened. The animals were collected from a pond near Cambridge, Massachusetts, during October and November, 1926. Horizontal lines showing 40 per cent and 70 per cent dead are drawn and the intersections of these lines with the curves give the times used in obtaining the points plotted in the graphs in Fig. 3.

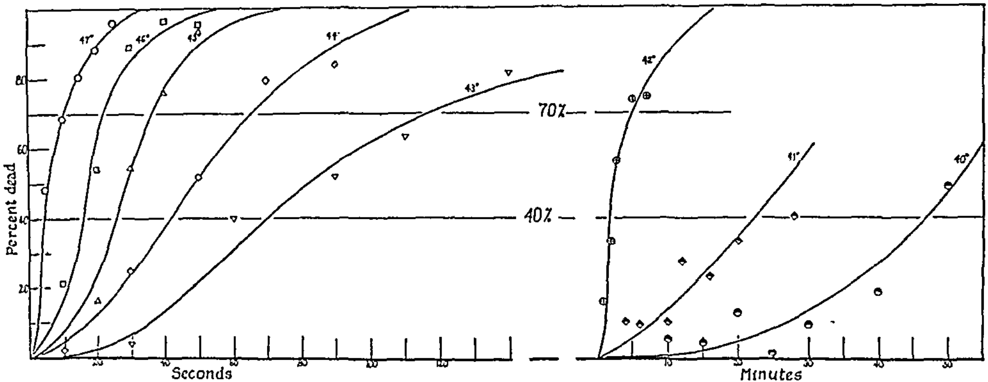

FIG. 2. Curves showing the velocities of killing of females of *M. macrocopa* at temperatures from 40° to 47°C. The females used in these experiments were all from one clone raised in the laboratory. The treatment of the data is similar to that for *D. pulex* (Fig. 1).

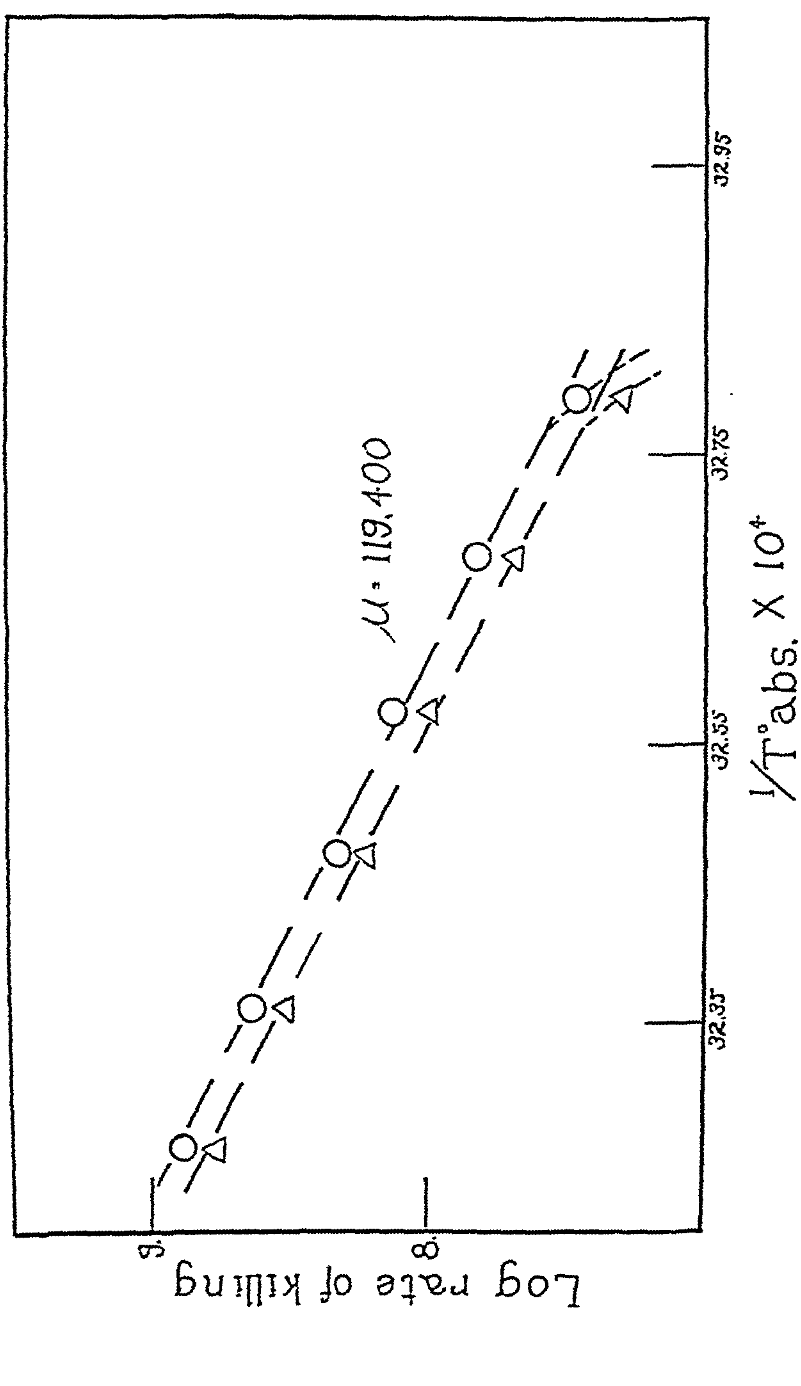

FIG. 3. *D. pulex.* Logarithms of the reciprocals of the times necessary to kill 40 per cent (circles) and 70 per cent (triangles) plotted against the reciprocals of the absolute temperatures. The slopes of the two lines are sensibly identical and yield $\mu = 119{,}400$.

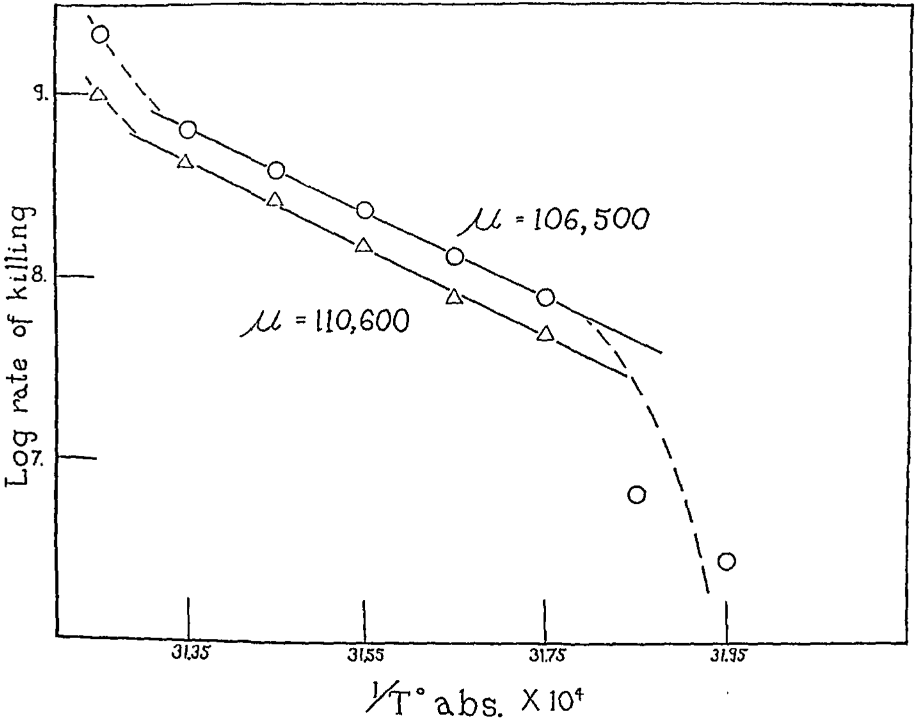

FIG. 4. *M. macrocopa*. Treatment as for *D. pulex* in Fig. 3. The slope of the lines between 42° and 46° yields μ = 106,500 for 40 per cent dead and μ = 110,600 for 70 per cent dead. The points for 40 per cent dead at 40° and 41° show an abrupt transition to the curve of life duration at sublethal temperatures.

TABLE I.

Estimations of temperature characteristics from data of various observers upon processes involving thermal destruction. As has long been known, the values of μ are quite high. In such series of data, however, it is necessary to recognize that "breaks" of several kinds occur at the boundaries of temperature zones within which a fairly constant fit to the Arrhenius equation is obtained. In view of the nature of such cases it is not surprising to find that, with increasing temperature, at these breaks change may occur to a higher or to a lower value of μ (or to a zone in which the equation no longer holds). It is to be noted that in certain cases objection might perhaps be raised to the type of effect which has been used as the basis of measurement. For this reason we have not tabulated the result of von Transehe's (1913) measurements, which for *D. magna* give a very high apparent μ (234,000, above 38°C.).

Material	Source	Value of μ	Temperature range
			°C.
Drosophila	Loeb and Northrop, 1917	107,900	31–35
Starfish, larvæ	Jacobs, 1919	160,000	32–36
Tubularia	Moore, 1910	116,200	29–36
		"	25–28
Sea urchin, larvæ	Loeb, 1908	93,600	27–32
		131,400	24–27
Botrytis	Smith, 1923	85,700	31–50
Sea urchin	Moore, 1910	72,800	36–42
" "		101,900	36–40
Bacteria	Bigelow, 1921	63,600	100–140
Ceramium	Ayres, 1916	27,700	28–38
Sea urchin, eggs ("viscosity")	Heilbrunn, 1924,*b*	114,800	32–37
Cumingia, eggs ("viscosity")	" "	64,000	32–43
Paramecium	Jacobs, 1919	34,600	36–40
		217,000	40–43
		106,000	36–40
Barley grains	Goodspeed, 1911	41,500	56–68
Wheat "	Groves, 1917	73,500	60–75
		20,300	75–87
" "	" "	53,000	70–92
" "	" "	48,400	60–80
		73,500	80–91
Pisum	Collander, 1924	94,500	35–55
Draparnaldia	" "	63,600	35–55
Elodea	" "	73,800	35–55
Beta	" "	94,500	40–60
Brassica	" "	94,500	40–60
Tradescantia	" "	73,800	40–65

error; the value of μ for 40 per cent dead is 106,500 while that for 70 per cent dead is 110,600 calories. The points for *M. macrocopa* give a slightly better fit than do those for *D. pulex*. This undoubtedly is due, first, to the fact that the death curves were based on larger numbers of individuals, and second, that all the individuals of *M. macrocopa* were from the same clone.

It is clear that within a certain range of temperatures the Arrhenius or Marcelin-Rice equation applies with some exactness. But it is also apparent that in the vicinity of a certain temperature there is fairly abrupt transition to the ordinary curve of life duration. We believe it desirable that such transition points be established, where possible, with all attainable exactness. For *D. pulex* this transition occurs near 31°C., as the animals will live and reproduce at 30° but die at 32°C. It will also be noted that the two points for 32°C. (Fig. 3) show a tendency to "fall off" in the same manner as is shown so strikingly in the graph for *M. macrocopa* (Fig. 4). The critical temperature for continued growth of *M. macrocopa* is much higher. Some females will live for days at a temperature of 40°C., and will reproduce normally at 39°C. The critical temperature for the continued life of this species must be in the neighborhood of 39.5° or 40°C.; at least, this holds when the animal is subjected to abrupt changes in temperature. At the uppermost temperatures used the "velocity of killing" shows abnormal acceleration. These features occur in similar data with other organisms.

IV.

The μ values here obtained may be compared with those computed for some similar cases. Several values of μ, and the sources of the data, are given in Table I. There is possibly a tendency for the values to be grouped around 70,000 and around 90,000 to 100,000; but the cases are too few, and the methods employed insufficiently uniform, to permit decision at present.

The killing of protoplasm by exposure to higher temperatures is accompanied not only by visible coagulative processes, roughly measurable as increased "viscosity" (Heilbronn, 1922; Heilbrunn, 1924, *b*), but, before these are well under way, by the structural alteration of the chondriome constituents (Policard and Mangenot,

1922; Fauré-Fremiet, 1925). Without the production of extreme effects, it is known that brief exposure at high temperatures may produce injury followed by hysteresis or by more or less incomplete recovery (*cf.* Wurmser and Jacquot, 1923; Klopp, 1924–25). It has been quite generally assumed that such disturbances are traceable to modifications of the colloidal state of protoplasm, and this interpretation permits certain views as to the process of thermal injury, as well as of the mechanism of adaptation to higher temperatures (*cf.* Jacobs, 1919). The coagulative process evident in connection with thermal killing involves proteins. The heat coagulation of protein follows the course of a first order process (Chick and Martin, 1910; 1912, *a*, *b;* Lewis, 1926, *a*, *b*, *c*) only when the pH is constant. During the coagulation of protoplasm this condition is pretty certainly not fulfilled, as a general thing.

The curve relating velocity of killing to temperature should therefore be expected to show a number of discontinuities, and it must be regarded as surprising that (over certain ranges of temperature) the agreement with the Arrhenius equation is in fact as good as it is. The velocity of heat coagulation of protein is governed by the velocity of the denaturing process, which Lewis (1926) shows to be hydrolytic. The typically high values of the temperature characteristic Lewis (1926, *a*, *b*, *c*) interprets as due to the additive nature of heats of activation, the hydrolysis of the protein which is preliminary to its flocculation being catalyzed at several linkages simultaneously (unless the possibility also be recognized that intramolecular changes may be responsible for quantitatively different cleavage mechanisms on either side of one or more transition temperatures). It might then be supposed that the observed critical increments should be integral multiples of that for a single cleavage. With comparable organisms it should be tempting to apply this notion to the temperature characteristics for thermal destruction. In the present case, the difference between the mean temperature characteristics pertaining to *Daphnia pulex* and to *Moina macrocopa* is 119,400 − 108,600 = 10,800, which may safely be rounded off to 11,000. If one ventures to regard this as perhaps signifying hydroxyl ion catalysis, the mean number of simultaneous linkage cleavages in the former case is 11, in the latter 10. There is no reason to suppose identical cleavage mechanisms in all

organisms, nor dependence of killing upon any one sort of protein even in a single cell. Hence a certain suggestive tendency of the perhaps best ascertained among known values of μ (Table I) to *aproach* a series with a common divisor of about 11,000 is probably illusory.

SUMMARY.

In spite of obvious possible sources of disturbance, the "velocity of killing" of organisms at supranormal temperatures, properly determined, tends to adhere to the Arrhenius equation for relation to temperature. Over certain ranges of temperature the relationship between *log velocity of killing* and $1/T°$ *abs.* is linear. Interpreted as due to the thermal denaturing of protein, it is possible that differences between the temperature characteristics for the killing process in closely related forms may be suggestive in regard to the mechanism of the denaturing. The temperature limits within which the linear relationships appear may be classed among those temperature levels which are critical temperatures for protoplasmic organization.

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ON THE NATURE OF THE EQUATION FOR GROWTH PROCESSES.

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I.

It was found by Bliss (1925–26) that there are certain deviations in the temperature characteristic when part of a given developmental period of *Drosophila* (prepupa) is passed at a lower temperature. If the equation used by Robertson (1923) to express the course of growth were correct, then an animal should pass corresponding portions of its development, as expressed in per cent of total developmental time, at any temperature that was favorable for development at all (Crozier, 1926–27). Just why this relation between temperature and partial times for development should be true in the case of Robertson's equation may be seen by an examination of the implications of the equation. Its differential form is

$$\frac{dx}{dt} = Kx\,(a - x),$$

where a is the initial endowment of a growth-promoting substance and x the amount formed after time t. A sigmoid curve results from the well known integrated form of this equation

$$\log \frac{x}{a - x} = k(t - t_1),$$

where $k = Ka$, and t_1 is the time at which the reaction is half completed; *i.e.*, when

$$x = \frac{a}{2}.$$

Since there is but one velocity constant (k), the growth curve for an animal raised at 25° should be made to coincide with the growth

curve of the same kind of an animal at 15° by bringing the two curves together at the end-point of development when $x = 100$ per cent, or some lesser value (*cf.* Crozier, 1926–27). But if the equation describing growth has more than one velocity constant and these constants are unequally modified by temperature changes, then the two curves for growth, when brought together at the end-point for development, will not coincide at all points along the time course of development. In other words, if an animal is allowed to develop for 50 per cent of the total normal time at 15°, and then is transferred to a temperature of 25°, it may take less time or more time to finish development than would be predicted on the basis of the fact that it has still to go 50 per cent of its total normal time at 25°. The differences between two such curves may be expressed in per cent gain or loss in time when a transfer is made from one temperature to another.

II.

A series of experiments of this type was made with *Pseudosida bidentata.* This species yields a constant value of the temperature characteristic ($\mu = 19{,}800$) between 14° and 28° (Brown 1926–27). The animals used were adult parthenogenetic females. These mothers, when about to release a brood of young, were isolated in test-tubes containing 25 cc. of culture water and the tubes were placed in a water bath. The bath set to operate at 25° was heated by an immersion heater, while the bath set to 15° was cooled below room temperature by means of the low temperature thermostat described by Crozier and Stier (1926–27). The control was in each case very good, as the temperature varied less than 0.01°C. The length of time required for the completion of an adult instar during these experiments was within the limits of the times determined for this species a year earlier (Brown, 1926–27) with somewhat less precise temperature control.

The average time for the completion of an adult instar (Table I) was obtained at 15° and at 25°. Then animals were allowed to develop at 15° for approximately $\frac{1}{4}$, $\frac{1}{2}$, and $\frac{3}{4}$ of the full time for completion of the instar at this temperature and then transferred to 25° and allowed to finish development (*i.e.*, until the release of the next brood). The reverse experiments were also made, by allowing the

animals to begin development at 25° and then making transfers to the lower temperature. The time gained or lost as the result of a transfer was computed as a percentage of the total time. The two theoretical curves (Fig. 1, C) which are drawn are separated from each other by an amount represented by this gain or loss. Of course it is not known that the curves are really sigmoid, but they probably are since most

TABLE I.

Apparent gain or loss in time when developing *Pseudosida bidentata* are transferred from 25° to 15° and from 15° to 25°. The "calculated total time" for development is the time passed at Temperature 1, plus the computed time for the remaining proportionate fractional time at Temperature 2. The gains and losses in time are given in the last vertical column as percentages of the total time, with their probable errors.

Condition of experiment	At 25°	At 15°	Actual total time	Calculated total time	Gain or loss in time
	hrs.	*hrs.*	*hrs.*	*hrs.*	*per cent*
Full time at 25°	46.0±.5				
33.1 hrs. at 25°, then transferred to 15°		35.4± .9	68.5	72.2	+5.0±.7
22.1 hrs. at 25°, then transferred to 15°		69.1± .8	91.2	94.6	+3.6±.5
11 hrs. at 25°, then transferred to 15°		108.0±1.7	119.0	117 1	−1.6±.4
34.3 hrs. at 15°, then transferred to 25°	35.7±.4		70.0	69.0	−1.3±.7
70.2 hrs. at 15°, then transferred to 25°	24 5±.8		94.7	93 0	−1.8±.5
105 hrs. at 15°, then transferred to 25°	13.2±.6		118.2	116.4	−1.5±.4
Full time at 15°		139.6± .4			

growth curves are of this shape, and at least the experimental points cannot be represented as falling on straight lines.

Two cases already published by others give data which may be compared with the foregoing. The curves shown in *A* of this figure are from data by Titschak (1925) for the pupal development of the clothes moth *Tineola biselliella* at 20° and 30°. Data are not given which would enable one to determine the presence or absence of a break in the graph for *log rate* against *reciprocal absolute temperature*

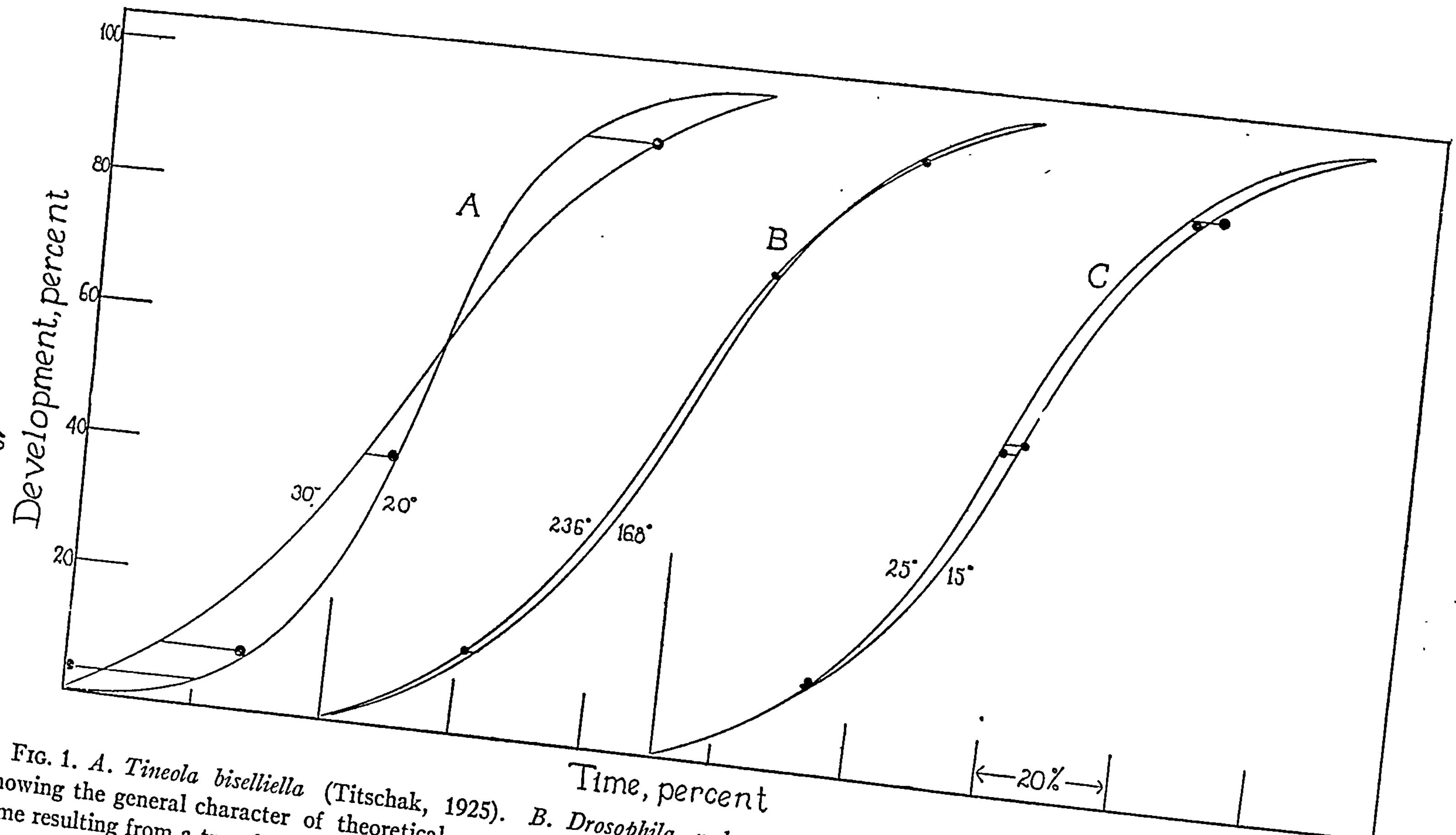

FIG. 1. *A. Tineola biselliella* (Titschak, 1925). *B. Drosophila melanogaster* (Bliss, 1925–26). *C. Pseudosida bidentata.* Graphs showing the general character of theoretical growth curves at two temperatures, as determined by the apparent gain or loss in time resulting from a transfer of developing animals from one temperature to the other. The curves are brought together at 100 per cent development by expressing the time in per cent. The horizontal lines and the symbols represent experimental transfers; those drawn to the left are losses in time and those to the right gains in time. The diameters of the symbols approximate the probable errors of the observations.

between these temperatures. The middle pair of curves (B) in this figure are from the data of Bliss (1925–26) for the prepupal development of *Drosophila.* These data were very carefully collected and the differences while small are statistically significant. In the graph for *P. bidentata* (C), as is also approximated in the two other cases, the diameter of the symbols represents the probable error of the observations. Of especial significance is the fact that the two transfers near 50 per cent on the time axis give approximately the same difference, but one (15–25°) represents a loss of time while the other represents a gain.

III.

The fact that it is impossible to make such pairs of curves coincide throughout, means that the growth equation of Robertson (1923) cannot hold; but that an equation with at least two velocity constants, which are unequally affected by temperature, must apply. Crozier (1926–27), in the paper referred to, has used such an equation, which is also apparently the correct one for a first order process with positive autocatalysis. The differential form of this equation is

$$\frac{dx}{dt} = (K_1 + K_2x)\,(A - x),$$

where K_1 is the velocity constant proper to the reaction $A \rightarrow x$, but in the absence of the catalytic effect of x, while K_2 is the velocity constant associated with x as a catalyst. The velocity of the formation of x will pass through a maximum (inflection point of the growth curve) when

$$x = \frac{K_2A - K_1}{2K_2}.$$

This equation was found to apply to the complete growth cycle of another species of cladoceran (Brown and Crozier, 1927–28), and presumably applies to the course of development within a single adult instar.

The integral form of the differential equation is

$$t = \frac{1}{K_1 + K_2A}\,ln\,\frac{A(K_2x + K_1)}{K_1(A - x)}.$$

The curve of this equation is sigmoid, but the inflection point depends on the ratio between the two velocity constants, and the resulting curves may be decidedly asymmetrical.

Results of the type indicated in Fig. 1 may be taken to prove that the curve describing the course of a developmental epoch must contain at least two velocity constants, and the equation proposed (Crozier, 1926–27) has made it possible to predict that the effects of changing temperature during development should be of the sort actually found. It was implied in the original account of this equation that such effects might be employed for the mapping out of the curves for processes otherwise "unseen." It is also obvious that these effects are of major significance for the understanding of the influence of fluctuating environmental temperatures.

SUMMARY.

An analysis of the growth curves of a cladoceran for one adult instar at each of two temperatures is made by comparing the apparent gains or losses in time when the animals are transferred from one of these temperatures to the other during the course of the developmental period. Since the curves for the two temperatures when brought together at their end-point do not coincide, the equation used to describe growth must have at least two velocity constants unequally affected by changes in temperature.

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KINETICS OF THE SWELLING OF CELLS AND TISSUES.

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Most cells or tissues when placed in distilled water increase in size owing to the entrance of the water into the cells and this swelling has frequently been compared to the swelling of simpler systems such as gelatin blocks. In preceding papers (1) formulæ have been derived which predict the course of the swelling of gelatin or the rate of entrance of water into a collodion membrane in a satisfactory way. If the swelling of plant or animal cells and tissues is really analogous to the swelling of gelatin or to the increase in weight of a membrane containing an osmotically active solution, it might be expected that the same formulæ could be used to predict the rate of swelling of cells or tissues. In the present paper data on the swelling of *Arbacia* eggs and on the swelling of slices of carrots or potatoes have been found to agree quite well with the formulæ referred to above.

Swelling of Arbacia Eggs.

The swelling of fertilized and unfertilized *Arbacia* eggs when placed in sea water diluted with tap water was studied by Lillie (2) who measured the increase in diameter of the eggs and computed the increase in volume. The results calculated to cc. are shown in Fig. 1 in which a smooth curve has been drawn through the experimental points. The figure shows that the fertilized eggs and the eggs on which fertilization membranes have been produced swell much more rapidly than the unfertilized eggs and soon reach a maximum. As Lillie points out, the increase in volume of the eggs should be proportional to the dilution of the sea water in which they are placed, if they are simple osmometers. Since the sea water was diluted $2\frac{1}{2}$ times the eggs should increase in volume $2\frac{1}{2}$ times. However they only double in size. Since the eggs

contain probably 20 to 30 per cent dry weight, and since it is the water content which should theoretically increase in proportion to the dilution of the sea water, this discrepancy would be partially corrected for if the calculation were made on a water basis rather than on the total volume. The expected increase in volume would also be less if the membrane were sufficiently elastic to oppose the entrance of the water. In the experiments of Lucke and McCutcheon (3), to be discussed later, it was found that the increase in size was nearly equal to that expected from the dilution of the sea water. It seems probable therefore that the force required to stretch the egg membrane is small and

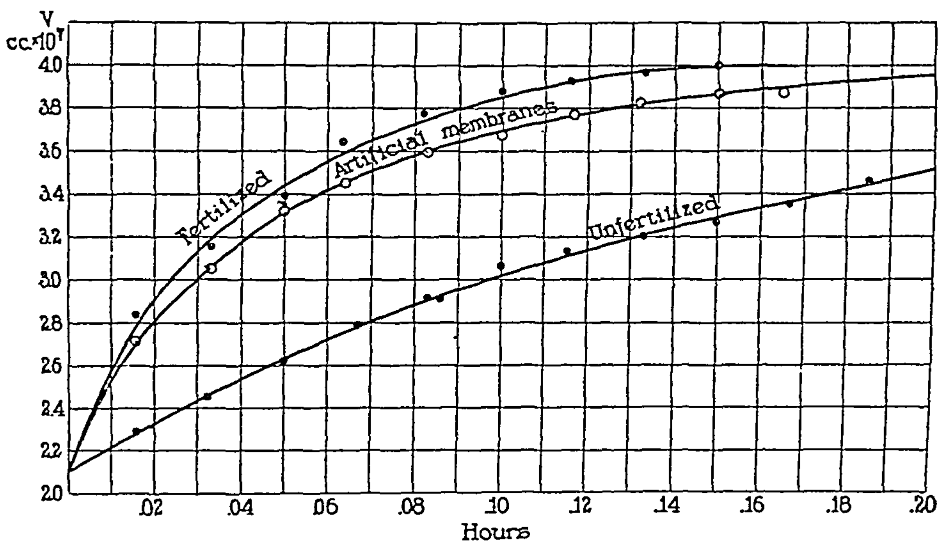

FIG. 1. Swelling of *Arbacia* eggs in 40 per cent sea water.

it has therefore been neglected in the following calculation. It has also been found by trial calculations that corrections for this elasticity or for the dry weight content of the egg fail to affect the results by more than the experimental error.

Lillie clearly recognized that the entrance of the water was a purely osmotic phenomenon, and that the rate was regulated by the permeability of the membrane and by the difference in the osmotic pressure of the inside and outside solutions. He assumed that all the other factors except the osmotic pressure difference were constant and therefore calculated the results on the basis of diffusion. He assumed, as

have other workers, that the rate of diffusion under these conditions should be proportional to the difference in pressure or,

$$\frac{dv}{dt} = K(P_e - P_m).$$

Lillie, in conformity with other workers, writes the integral of this equation as

$$K = \frac{1}{t}\, ln \frac{P_e - P_m}{P_t - P_m}$$

in which P_e is the initial pressure in the egg, P_m the pressure of the external solution and P_t the pressure at time t. It is then assumed that since the pressure is inversely proportional to the volume, the equation becomes

$$K = \frac{1}{t}\, ln \frac{v_{eq} - v_o}{v_{eq} - v_t}$$

where v_{eq} is the equilibrium volume. It appears to the writer, however, that in order to integrate the equation given above it is necessary to express P in terms of v *before* integrating. Substituting $\frac{P_o}{v}$ for P_e in the differential equation and adding the terms for the area and thickness of the membrane, the equation becomes

$$\frac{dv}{dt} = \frac{CS}{h}\left(\frac{P_o}{v} - P_s\right),$$

or

$$C = \frac{hv_e}{SP_o t}\left(v_o - v_t + 2.3\, v_e \log \frac{v_e - v_o}{v_e - v_t}\right), \tag{1}$$

in which S is the surface of the membrane, P_o the "osmotic constant" of the solution inside the membrane, P_s the osmotic pressure of the external solution (assumed constant), v_e the volume at equilibrium, v_o the volume at the beginning of the experiment, h the thickness of the membrane and C the diffusion or "permeability" constant (1). In the units used by the writer C represents the cc. of water which will pass through 1 sq. cm. of membrane 1 cm. thick in 1 hour under 1 mm. Hg pressure. The equation has various forms depending on whether the

surface, thickness, and elasticity of the cell, and the osmotic pressure of the external solution, are considered constant as in this case. If the membrane is considered to be elastic or if the pressure of the outside solution changes, another term must be added. This equation has been found to agree with the rate of osmosis through collodion membranes (1).

Lillie's experiments have been calculated by this equation and the results are shown in Table I. The monomolecular constants are given for comparison. The values used for the volumes have been interpolated from the smooth curve since this procedure brings out better any drift of the constant which may be obscured by accidental varia-

TABLE I.

Swelling of Arbacia Eggs in 40 Per Cent Sea Water after Lillie.

$v_s = 4.0 \times 10^{-7}$ cc. $v_0 = 2.1 \times 10^{-7}$ cc. $S = 1.7 \times 10^{-5}$ sq. cm. $P_0 = 4.6 \times 10^{-3}$ mm. Hg.

	Fertilized			Artificial membrane		
$v \times 10^7$	t	K_m	$\frac{C}{k} \times 10^5$	t	K_m	$\frac{C}{k} \times 10^5$
cc.	*hrs.*			*hrs.*		
2.5	.0080	12.9	3.52	.0095	10.9	2.98
2.7	.0130	12.7	3.62	.0160	10.3	2.97
2.9	.0200	11.9	3.57	.0240	10.3	2.97
3.1	.0290	11.2	3.52	.0350	9.3	2.91
3.5	.0550	10.5	3.66	.070	8.4	2.92

tion in the case of the experimental points themselves. The table shows that the monomolecular constant decreases as Lillie stated in the case of the fertilized and artificial membrane eggs, while equation (1) gives constant values.

The entrance of water into the eggs therefore follows the same course as the entrance of water into collodion membranes. In the case of the collodion membrane there is no increase in size of the membrane, and the surface and thickness are therefore constant. The eggs however increase in volume and the surface therefore also increases. It might be expected therefore that this factor would have to be considered, and that an equation derived on the assumption that

the surface was constant would not fit. Apparently, however, in the case of the fertilized eggs, this is not the case. The results indicate that the "diffusing surface" of the fertilized egg is not changed by the increase in volume or, if it is considered that the water enters through pores, that the number, radius and length of the pores is unchanged by the swelling.

The value of C/h is about 3×10^{-5}, which is about ten times smaller than the corresponding figure for collodion membranes (1). Since the egg membrane is probably less than one thousandth as thick as the collodion one, the permeability through the same thickness of membrane would be more than a thousand times as great for collodion as for the egg membrane. The results quantitatively confirm Lillie's statement that the egg membrane is relatively impermeable to water.

Unfertilized Eggs.

The course of the swelling of unfertilized eggs follows the monomolecular formula quite closely in Lillie's experiments. The swelling of unfertilized eggs has been studied in detail by Lucke and McCutcheon (3), and these workers also state that the swelling follows the monomolecular formula. Recalculation of their results shows a slight rise in the monomolecular constant in most cases (which however is probably hardly outside the experimental error), while the constant of equation (1) shows a definite increase. If the correction is made for the dry weight content of the egg, this increase in the monomolecular constant is slightly greater but even then is not marked. As stated above, this increase in the rate might be expected owing to the increase in surface of the egg. This effect can be taken into account in two ways, both of which however lead to the same equation. If the volume of the membrane is assumed to remain constant and the rate of diffusion is assumed proportional to the surface and inversely proportional to the thickness of the membrane, then, since the surface times the thickness equals the volume (which has been assumed constant), $S/h = 25v^{\frac{4}{3}}/m$, where m is the volume of the membrane. If, on the other hand, the water is supposed to flow through pores in the membrane, it may be assumed that the increase in surface during swelling is due to the increase in the size of the pores, and that the solid membrane surface remains practically constant. The surface of

the pores will be proportional to $5v^{\frac{2}{3}}$, and since according to Poiseuille's law the rate of flow through fine capillaries is proportional to the fourth power of the radius or to the square of the surface, the rate will be proportional again to $25v^{\frac{4}{3}}$.

The differential equation for the rate of swelling, then, neglecting the elasticity of the membrane and assuming that the osmotic pressure in the egg is inversely proportional to the volume of the egg, is

$$\frac{dv}{dt} = 25C_2\, v^{\frac{4}{3}} \left(\frac{P_o}{v} - \frac{P_o}{v_e}\right)$$

or, on integration,

$$C_2 = \frac{v_e^{\frac{2}{3}}}{25P_o t}\left[\left[1.15 \log \frac{v_e^{\frac{2}{3}} + v^{\frac{1}{3}} v_e^{\frac{1}{3}} + v^{\frac{2}{3}}}{(v^{\frac{1}{3}} - v_e^{\frac{1}{3}})^2} + \sqrt{3}\tan^{-1} - \frac{2v^{\frac{1}{3}} + v_e^{\frac{1}{3}}}{v_e^{\frac{1}{3}}\sqrt{3}}\right] - \left[1.15 \log \frac{v_o^{\frac{2}{3}} + v_o^{\frac{1}{3}} v_e^{\frac{1}{3}} + v_o^{\frac{2}{3}}}{(v_o^{\frac{1}{3}} - v_e^{\frac{1}{3}})^2} + \sqrt{3}\tan^{-1} - \frac{2v_o^{\frac{1}{3}} + v_e^{\frac{1}{3}}}{v_e^{\frac{1}{3}}\sqrt{3}}\right]\right], \qquad (2)$$

in which the second term represents the integration constant.

The results of some of Lucke and McCutcheon's experiments at different temperatures are given in Fig. 2, and the constants calculated by the monomolecular equation and by equation (2) in Table II. The monomolecular constants show a slight rise in most cases, while the constant of equation (2) shows in some cases a decrease.

It may be noted that if the water is supposed to flow through holes in the membrane then the constant C_2 contains the viscosity of water and should be corrected for the viscosity of water before comparing the values at different temperatures. It should also be corrected for the effect of temperature on the osmotic pressure, but since this is proportional to the absolute temperature this correction may be neglected for small changes in temperature. When the viscosity correction has been made the values of C given in the last row of Table II are obtained, and show a small and irregular temperature coefficient. The values of C in this equation cannot be compared directly with those obtained in equation (1), since they contain another constant representing either the volume of the membrane or the ratio of total surface to pore surface depending on which mechanism is assumed to be at work.

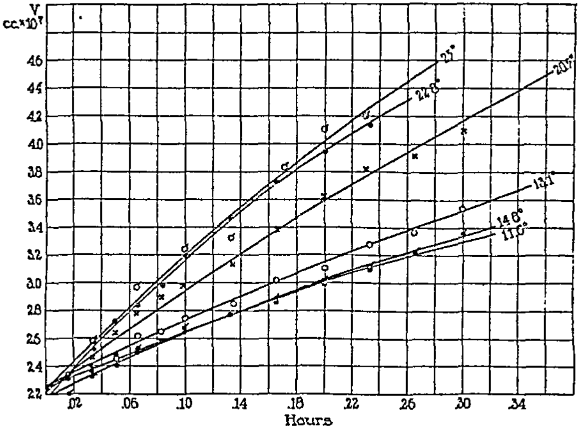

FIG. 2. Swelling of *Arbacia* eggs in 40 per cent sea water at different temperatures.

TABLE II.

Summary. Monomolecular and Osmotic Pressure Constants.

$v_o = 2.20 \times 10^{-7}$ cc. $v_e = 5.0 \times 10^{-7}$ cc. $P_o = 4.7 \times 10^{-3}$ mm. Hg.

	11°		13°		14.8°		20.5°		22.8°		25°	
$v \times 10^7$	K_m	$C_1 \times 10^3$	K_m	$C_1 \times 10^3$	K_m	$C_1 \times 10^3$	K_m	$C_1 \times 10^3$	K_m	$C_1 \times 10^3$	K_m	$C_1 \times 10^3$
cc.												
2.6	.82	1.50	.97	1.81	.77	1.42	1.38	2.54	1.8	3.22	1.55	3.47
3.0	.77	1.41	.93	1.69	.78	1.41	1.42	2.62	1.86	3.35	1.69	3.45
3.4			.96	1.67	.79	1.38	1.50	2.62	2.0	3.49	1.84	3.55
3.8								2.67		3.54		3.66
$C_1 \times 10^3 =$		1.45		1.72		1.40		2.60		3.40		3.53
$\eta \times 10^2 =$		1.30		1.22		1.14		1.00		.95		.89
$C \times 10^5 =$		1.9		2.1		1.6		2.6		3.2		3.1

Effect of the Concentration of the Sea Waters.

Lucke and McCutcheon (3) also obtained some interesting results in connection with the effect of the concentration of sea water on the rate of swelling. They found that when the monomolecular constants for the process were compared, the constants decrease rapidly as the concentration of sea water in which the eggs were placed decreased. The permeability of the eggs to water was therefore apparently much less in dilute than in concentrated sea water. According to the mechanism of the process outlined above, the "permeability constant" C_2 is approximately proportional to the monomolecular constant times the volume at equilibrium, *i.e.*

$$C \backsim K_m v_e.$$

TABLE III.

Rate of Swelling of Arbacia Eggs in Various Concentrations of Sea Water.

Per cent sea water	80	60	40	20
K	.072	.024	.012	.006
$v_e \times 10^9$ cc	240	350	500	1050
Kv_e	17.3	8.4	6.0	6.3

When the values of K_m obtained by Lucke and McCutcheon are corrected in this way the values shown in Table III are obtained. They still show that the membrane is less permeable in 20, 40 and 60 per cent sea water than in 80 per cent, but the differences are not so marked as is the case with the monomolecular constant itself.

Swelling of Plant Tissue.

In the case of *Arbacia* eggs just considered the resistance to the entrance of the water is localized at the membrane, and in confirmation of this it was found that the kinetics of the reaction is analogous to that of water entering a collodion bag. In the case of material like gelatin, however, it is evident that the entire mass offers resistance to the passage of the water and that the resistance is not confined to the surface layers. It would be expected therefore that the swelling curves for

gelatin would be quite different from those for the passage of water into collodion sacs, and this was found to be the case. The swelling of slices of carrot or potato as studied by Stiles and Jørgensen (4) should presumably follow approximately the same course as that found for gelatin, since the structure of such tissues is very similar to that assumed for the gelatin.

Stiles and Jørgensen found that slices of potato or carrot neither lost nor gained weight when placed in M/4 sugar or in M/8 NaCl, lost weight in more concentrated solutions and gained weight in more dilute solutions. They assume that the tissue is permeable only to water under these conditions. The results of these experiments are shown

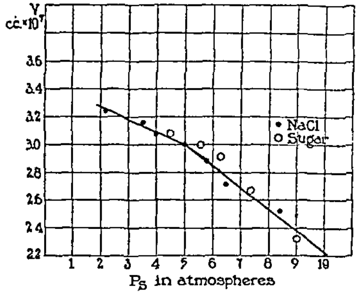

FIG. 3. Swelling of slices of potato in salt or sugar solutions.

in Fig. 3, in which the osmotic pressure of the NaCl in atmospheres as calculated from the freezing point depression (5) has been plotted against the volume of the potato slices expressed as gm. water per gm. dry weight. If it is assumed that the tissue is an elastic body, and that the water is drawn in by the difference in the osmotic pressure of the solutions inside and outside of the tissue, and further that this osmotic pressure is inversely proportional to the water content of the tissue, then the equilibrium condition will be defined (1) by the equation

$$\frac{P_e}{V_e} - P_s = \frac{K_s(V_e - V_f)}{V_f},$$

where P_o is the osmotic constant of the inside solution (*i.e.* $\frac{P_o}{V_o}$ equals the osmotic pressure of the salt solution which causes no change in volume), P_s is the osmotic pressure of the salt solution in which the tissue is immersed, V_e is the volume of water per gm. dry weight at equilibrium, V_f the volume when under no strain and K_e the bulk modulus. The values of K_e calculated from the data are shown in Table IV. Pressure which tends to remove water is called negative, and pressure required to add water, positive. The table shows that the value of K_e, which may be defined as the pressure in atmospheres required to change the volume by an amount equal to the volume of

TABLE IV.

Swelling of Potato in Various Concentrations of NaCl.

$V_f = 3.00 \quad P_o = 15.00$

$V = \frac{\text{gm. } H_2O}{\text{gm. dry wt.}}$	NaCl Moles per 1000 gm. H_2O	Osmotic pressure NaCl Atmospheres	K_e Atmospheres
2.56	.20	8.4	17
2.72	.16	6.5	10
2.88	.143	5.8	13
3.04	.125	5.0	
3.08	.10	4.0	35
3.16	.083	3.5	25
3.24	.050	2.2	33
3.68	.000	0	18

water originally present, is about 14 when the water is removed and about 30 when water is added. The values of K_e show quite large variations, but they are within the experimental error (except for the value in distilled water), since the equation is of such form that small errors in the experimental value are greatly exaggerated in calculating the value of K_e. In the case of distilled water the value suddenly decreases; that is, the tissue swells more than would be expected from the other results. This is the usual result with elastic substances when the elastic limit has been exceeded. The figures show that a very considerable force is required to either increase or decrease the size of the tissue and that as might be expected less force is required to contract than to expand it.

Kinetics of the Swelling Process.

The rate at which water enters the tissue was also determined by Stiles and Jørgensen. In the case of potato there seems to be some doubt as to whether a true equilibrium value is reached in distilled water, and the writer was unable to assign any very definite value to this equilibrium volume from the data published. The data for the swelling of slices of carrot, however, at various temperatures, show a definite maximum and these experiments have been used. Stiles and Jørgensen's results are shown graphically in Fig. 4, in which the increase in weight of slices of carrots in distilled water at 10°, 20° and

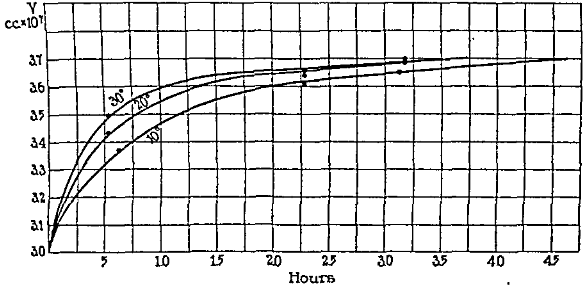

FIG. 4. Swelling of slices of carrot in distilled water at various temperatures.

30° expressed as gm. water per gm. dry weight has been plotted against the time in hours.

Stiles and Jørgensen considered that the water entered the cell owing to osmotic forces, and that this entrance was opposed by the elasticity of the tissue. They also point out that the process was probably analogous to the swelling of simpler systems such as gelatin blocks, so that the present treatment does little more than express their conclusions in mathematical form.

If the water is assumed to flow through small pores in the tissue (in this case possibly the spaces between the cells), in order to reach the

cells away from the surface then the rate of flow through the tissue as a whole will be expressed by Poiseuille's law

$$\frac{dv}{dt} = \frac{Cr^4 SP}{\eta h},$$

in which v is the volume of water, r the radius of the pores, S the surface of the piece of tissue (assumed proportional to the number of pores), P the swelling pressure, η the viscosity of water at the temperature used, and h the distance through which the water has to diffuse. C, the "permeability constant," will therefore be the cc. of water that will flow through 1 sq. cm. of tissue 1 cm. thick in 1 hour under 1 mm. Hg pressure. In the case of gelatin it was found that the rate of flow of water was less in swollen than in unswollen blocks, so that r^4 was assumed inversely proportional to V. In the case of slices S is constant and h is equal to $\frac{V}{2S}$, since the water enters from both sides and the average distance traversed by the water will be $\frac{1}{4}$ the thickness of the block. The swelling pressure will be equal to the difference between the osmotic pressure and the elastic force or

$$P = \frac{P_o}{V} - \frac{K_e(V_e - V_f)}{V_f}.$$

In order to express the results as volume of water per gm. dry weight, let $v = Vg$ and hence $dv = gdV$ where v is the total volume of water, V is the volume of water per gm. dry weight and g is the weight of solid material. Substituting these values the differential equation becomes

$$\frac{dV}{dt} = \frac{2\,C\,S^2\,P_o\,(V_e - V)\,(bV + V_f)}{V^3\,V_f\,V_e\,g^2},$$

the integral of which is:

$$\frac{C}{\eta} = \frac{g^2\,V_e\,V_f}{2\,P_o\,S^2t}\left[\frac{V_o^2 - V^2}{2\,b} + \frac{(b\,V_e - V_f)\,(V_o - V)}{b^2} + \frac{2.3\,V_e^3}{(b\,V_e + V_f)}\log\frac{V_e - V_o}{V_e - V}\right.$$
$$\left. - \frac{2.3\,V_f^3}{b^3\,(b\,V_e + V_f)}\log\frac{V_f + b\,V}{V_f + b\,V_o}\right],$$

in which $b = \dfrac{K_eV_e}{P_o}$.

In this case V_o is the same as V_f since it is assumed that the tissue is under little or no strain before being placed in water. Table V shows the values of C calculated from the experiments by means of this formula. The last row gives the value corrected for viscosity. The table shows that the value is fairly constant for the different times, and also that there is little or no effect of temperature. This may be considered as indicating that the water does flow through fine pores and that the temperature coefficient is therefore that of the viscosity of water.

The foregoing results indicate that the formulæ derived for the swelling of gelatin may be used at least as a first approximation for

TABLE V.

Swelling of Slices of Carrot in Distilled Water at Various Temperatures.

$g = .45$ $P_o = 10.5 \times 10^3$ $V_o = 3.0$ $V_e = 3.70$ $K_e = 13 \times 10^3$ $S = 5.0$ sq. cm.

V	10° $\frac{C}{\eta} \times 10^4$	20° $\frac{C}{\eta} \times 10^4$	30° $\frac{C}{\eta} \times 10^4$
3.32	9.55	14 9	19.1
3.47	9.37	13.3	18.8
3.60	8.70	13.2	16.8
Average $\frac{C}{\eta} \times 10^5$	9.2	13.5	18.2
$\eta \times 10^2$	1.31	1.00	.800
$C \times 10^5$	12.0	13.5	14.5

the swelling of tissues and cells in certain cases. It may be pointed out that they cannot be used unless there is evidence that only water passes the membrane, that there is really a fairly constant bulk modulus, and unless the equation connecting the change in pressure with the increase in volume is known. In most of the experiments reported these conditions are not fulfilled. The swelling of *Xanthium* seeds for instance, studied by Shull (6), as well as results with other seeds, is difficult to interpret owing to the fact that the relation of the pressure to volume is not known and certainly cannot be assumed to be a simple inverse ratio as has been assumed for the present tissues which contain a very much larger amount of water originally. The writer has

found that by using an empirical exponential relation between the pressure and volume in *Xanthium*, the rate of swelling may be approximately calculated; but the method used really amounts to introducing two new arbitrary constants in the formula and deprives the results of their significance.

SUMMARY.

The rate of swelling of *Arbacia* eggs in dilute sea water, studied by Lillie and by Lucke and McCutcheon, may be expressed by the formulæ derived for the rate of increase in volume of a solution enclosed in a collodion sac.

The rate of swelling of slices of carrot in distilled water, measured by Stiles and Jørgensen, may be expressed by the equation derived previously for the swelling of similarly shaped blocks of gelatin.

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THE PRENATAL GROWTH OF THE MOUSE.

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The period before birth offers a unique opportunity for the study of mammalian growth. At no other period are the basic external influences, such as temperature, nutrition, and humidity, so accurately regulated; while the changes in speed, in relative magnitude, and in complexity are greater than at any other time. Yet the difficulties in the way of obtaining reliable data for this period are considerable. The rapidity of the changes, combined with the impossibility of determining the exact time of fertilization of a given ovum, necessitate large numbers; the dissection and handling of the smallest embryos is difficult and has seldom been attempted. Indeed the scarcity of data for this whole period explains a dangerous tendency to accept such data on their face value.

The data for man are subject to errors from pathological conditions of mother and embryo, as well as to gross inaccuracies in age. Averages for the rabbit are given by Friedenthal (1914), but these include weights of embryonic membranes and placentæ. The data for the rat are open to criticism which is presented later in detail. The most reliable figures are those for the guinea pig (Draper, 1920, and Ibsen, ms.). In spite of the extensive use of the mouse for studies on postnatal growth, no published data on its prenatal weight have been discovered by the authors. This paper presents such data from 115 litters, including 959 embryos, and also attempts to show a similarity in the progress of prenatal growth in certain vertebrates. Brody and Ragsdale (1922–23) and Brody (1925–27) have presented a method for finding the age equivalence for different animals based on the latter part of postnatal growth and upon conception age. Friedenthal (1914) had previously shown a similarity in the relative growth rates of different animals by plotting the logarithms of the weight against

the logarithms of the age. He emphasized the point that the similarity appeared only when the age was calculated from the time of conception. On the other hand, we are led to the conclusion that the age of embryos should be counted from the beginning of the embryo proper. This method reveals a closer harmony among curves of prenatal growth than has been previously demonstrated, and shows that the major differences in the curves of the mouse, the guinea pig, and the chick lie in the amount of tissue involved in the first organization of the embryo and in the length of the prenatal life.

Embryo Age.

In mammals, the separation of the egg from the main food supply is correlated with a precocious development of the trophoblastic elements of the morula. These form the yolk sac and the traeger, which effect the first connection with the maternal food supply. They are well developed before the visible organization of the embryo is started. This preliminary stage occupies an appreciable part of the gestation period. If natal and prenatal growth are to be compared, the embryonic membranes and placentæ must be excluded; hence the growth curve of the embryo should start with the beginning of the embryo proper and not include the preliminary stage or the extraembryonic tissues. This proposition is shown to be justified by the data themselves.

Any definition of the beginning of the embryo proper must be largely arbitrary; as a practical criterion we propose the primitive streak stage. This establishes the main axis of the embryo and includes the differentiation of the anlage of the embryo proper. The time of its occurrence can probably be determined with as great accuracy as can the time of the fertilization of the ovum. The time between conception and the primitive streak stage is sufficiently long to make a considerable difference in the mathematical description of the embryo growth curves. And this difference is great enough to afford a statistical method that may make it possible to estimate the time of the primitive streak stage from the growth curve of the embryo. This method is shown to hold good for the mouse, the guinea pig, and the chick.

Material and Methods.

Ancestry of Embryos Weighed.—The maternal grandmothers of the embryos came from the Bagg albino strain which has been inbred since 1913, and for the last twelve generations in our colony the inbreeding has been between brother and sister. This strain was chosen because of its good breeding and nursing qualities. It carries the genes for homozygous brown agouti self-color, dark eye, and intense pigment. The females used were over 3 months old and they had not nursed young for at least 3 weeks before this mating. The maternal grandfathers of the embryos came from the Storrs-Little strain which originated from the Little dilute brown strain, inbred since 1909, by one out cross with back crosses to the pure dilute brown strain for five successive generations. This strain has been inbred brother to sister for ten generations. The animals are pink-eyed dilute brown self-colored. The mothers of the embryos, F_1 hybrids between the two lines just described, were nursed in litters cut down to six at birth. They were weaned when 4 weeks old and held in groups of six or less in mating boxes until over 3 months old before being mated. All embryos came from first litters. Their fathers, which were over 3 months old at the time of fertilization, came from a line (No. 89) of intense brown agouti self-colored animals which has been inbred in very large numbers in this colony, brother by sister, for nine generations.

The highly inbred lines were desired to give uniformity; but as the greatest vigor was also desired, hybrid mothers were used (Wright, 1922), with the hope of stabilizing litter size and reducing prenatal mortality. The fathers were taken from a third strain to reduce the segregation as far as possible. First litters were used because in these the prenatal mortality has been found to be the lowest. Breeding was held off until 3 months old to permit full body development, since hybrid embryos are frequently too large for an incompletely grown mother.

Timing Copulations.—The males were kept isolated in small boxes in front of the large boxes of females. For copulation the males were put into the boxes with the females for an hour each day, then all the females were examined for vaginal plugs with the aid of a pure silver probe. As soon as a plug was discovered the female was isolated in a small box where she was left without being touched again until the time assigned for taking her embryos.

Diet and Care.—Bread, fresh milk, canary seed, hemp, pin-head oatmeal, supplemented with cabbage twice a week, constituted the diet. The boxes were provided with shavings and cotton batting, and with the exception of those containing pregnant females, were cleaned every week. The boxes were kept in an equably heated laboratory.

Dissection of Embryos.—The embryos were taken during the months of November to April. The pregnant females were killed mechanically at the end of some multiple of 24 hours from the end of the hour the male was last with the female. This was accurate within a few minutes. The 19 day embryos are not included. Since most litters are born before the end of the 19th day, those not yet born are selected on the basis of prolonged gestation, and it is possible that a correlation

exists between the extended gestation and the condition of the embryos. Until such a correlation is disproved, curves of prenatal growth should exclude data beyond the minimum period of gestation.

Immediately on killing the mother the uterus was exposed and cooled; this suddenly reduced the vital processes of the embryos to a minimum and delayed actual death for a considerable time. Even 3 hours after the death of the mother, the hearts of the early embryos would pulsate if warmed. All degenerate or recently dead embryos were excluded. The heart beat was the best sign of life; in later embryos, the movements of the skeletal muscles. The general body color was markedly different in the rare cases of very recent death.

Embryos 13 to 18 days were removed under a Leitz binocular microscope magnifying 7.5 diameters. When removed from uterus and membranes, the umbilicus was clamped with forceps to reduce bleeding and severed close to the abdomen. A small glass spoon was used for lifting the embryo; after the excess fluid was blotted off, it was placed in a glass ring between cover-glasses, moved to the balance shelf on a convenient carrier, and lifted to the balance pan with forceps.

The membranes of embryos of 10 to 12 days were removed in Locke's solution under the Leitz binocular, lifted out of the solution to the cover-glass with the glass spoon, there freed from excess fluid with pointed rolls of paper toweling, the ring put in place and covered with the second cover-glass.

The whole decidual capsule for 8 to 10 day embryos was opened in Locke's solution by a single equatorial cut with fine scissors. This permitted the embryo to slip out from the decidua capsularis. It was then pipetted to a second container with a black paraffin bottom and fresh Locke's solution. With specially ground, hook-pointed needles and brilliant illumination under a Greenough binocular magnifying 30 diameters, the amnion, allantois, and remainder of the yolk sac were removed from the embryonic area. Cutting against the paraffin held the embryo in place until the dissection was completed. In many 8 day embryos the separation of the membranes had to be entirely by needle cutting, but in the 9 day embryos the membranes could be gathered between the blades of a fine pair of iridectomy scissors and nicely removed with two cuts. This dissection was exceedingly slow, 3 hours usually being required for a litter. When prepared, the embryos were pipetted directly to the cover-glass and the drop of fluid removed with the paper pointers while under observation with the high power binocular; then the ring and the second cover-glass added.

While two of the authors shared in removing the embryos older than 12 days, the younger ones were all dissected by the same person, thus ensuring a high degree of uniformity in the details of technique.

Glassware.—The glassware included numerous sets of five different sizes. Each piece of glass was etched with an identifying mark. Two sets of glass were used alternately for successive embryos. After an embryo was weighed, the glass was washed in water, rinsed in alcohol, and dried with a lintless silk cloth. The

weight of each set of glass was determined, after being washed in this manner, every day it was used.

Balances.—For 10 to 18 day embryos a Sartorius balance was used, which, in the hands of the observer who made all the readings, gave an accuracy within 1/10th mg. Eimer and Amend analytical weights, with whole gm. gold plated, were used with this balance. Embryos of 8, 9, and a few of 10 days were weighed on a Troemner Assay balance (new No. 30) with Troemner triple checked Precision button weights. The total load prescribed for this balance is 1.0 gm., but this was increased to over 1.5 gm. by substituting cover-glasses for the balance pans. The greatest load used was 0.3 gm. In actual practice this balance proved to be accurate within 1/100th mg. Both balances were provided with hinged side doors which were used exclusively in the process of weighing. Both pointer scales were read with a magnifying glass. The balances were kept permanently in position on a special rigid shelf bolted to a masonry wall that is subject to no sensible vibrations. When not in use each balance was kept inclosed in a case consisting of hinged top and sides fastened to the wall and operating free of the shelf without jarring the balance. All weighing was done within 6 months while the steam heat was on and the windows mostly closed (November 3, 1926, to April 17, 1927).

Weighing.—Embryos 9 to 18 days were weighed individually. While one was being weighed the next one was being dissected, and the work so timed that as soon as the second cover-glass was in place the embryo was immediately transferred to the balance pan. Embryos awaiting dissection were left in their intact amnions in the uterus. Embryos from two 8 day litters were weighed individually, but the other 8 day embryos were weighed in groups.

Zero was determined once a day on the Sartorius balance from five sets of five pointer readings. On the Troemner, five pointer readings were recorded before and after each embryo weighing and the average between these two deviations was used to correct the embryo weight for zero. All glassware was weighed by the overload method. For weights of all embryos under 18 days the last place was determined by one set of five pointer readings from which the deviation was calculated, corrected for zero, and divided by the sensibility constant. All pointer readings are on file.

Histological Method.—The embryos used for sectioning were fixed in either Bouin's fluid or Allen's B_{15}, and run carefully up to paraffin. Some were sectioned transversely and others longitudinally.

RESULTS.

The average weights and the frequencies for each day appear in Table I. The distribution of individual weights for each day is shown in Fig. 1. In order to include all the data in one legible chart, the logarithmic scale is used for the weights. And in order to present graphically the number of individuals of each weight on each day, a

uniform scale is used for the abscissa, which thus gives both age and number of individuals. This chart shows the curve of the calculated means, the observed means, the modes, the ranges, and the classified distributions of individual weights for each day.

The distributions show: (1) that for each day individuals are found equal in weight to the mode of the day before. The regularity of this overlap may be considered evidence of as much as 24 hours variation in the interval between copulation and the fertilization of the ova. (2) Modes and means are generally close together. (3) The highest

TABLE I.

Weights of Mouse Embryos (See Text for Ancestry of Material).

Conception age t	Embryo age $t - 7.2$	Frequencies: Litters	Frequencies: Embryos	Average weight: Observed $\Sigma W/n$	Average weight: Calculated $.0000000456(t - 7.2)^{3.619}$
8	.8	11	93	.00008	.00009
9	1.8	8	76	.00147	.00173
10	2.8	10	88	.00860	.00869
11	3.8	10	101	.0329	.0265
12	4.8	11	77	.0762	.0622
13	5.8	11	100	.1298	.1240
14	6.8	11	74	.2288	.2213
15	7.8	10	75	.3651	.3656
16	8.8	12	95	.5926	.5677
17	9.8	11	89	.8467	.8408
18	10.8	10	91	1.190	1.198
Totals..............		115	959		

individual weights on each day form a curve nearly parallel to that of the means. (4) The means do not approximate a straight line.

Brody (1926–27)[1] shows that a straight line may be drawn through Stotsenberg's (1915) averages for the rat when these are plotted on semilogarithmic paper as are the mouse averages in Fig. 1. This means either that the rat and mouse follow essentially different laws of prenatal growth, or that the two sets of data are not equally reliable. Although these rat data have become familiar in the literature, their

[1] Brody (1926–27), Fig. 9, p. 650.

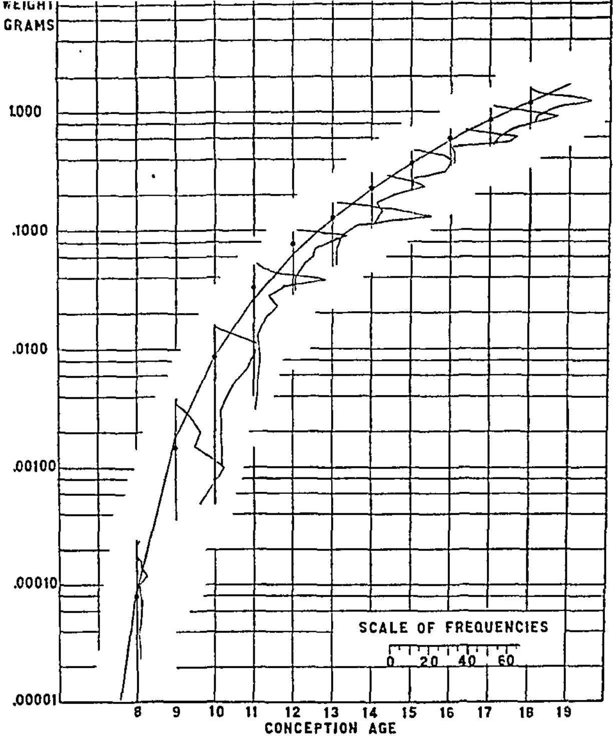


FIG. 1. Logarithmic distributions of classified wet weights of individual mouse embryos 8 to 18 days from conception. Ordinates, in logarithmic ruling, give actual weights in gm.; abscissæ in arithmetic ruling give age and number of individuals. On each day the range of individual unclassified weights is shown by a vertical line which is used as the base line for the frequency distribution of classified individual weights. The number of cases in the distribution is shown by the distance to the right of points connected by light lines; note scale of frequencies at bottom of chart. The means, weighted by the number of individuals in each litter, are shown by dots on the vertical base lines. The theoretical means for each day are calculated from the formula

$$\log W' = 3.649 \log [10 (t - 7.2)] + \bar{8}.6587$$

and are connected by a continuous line. The mean for the 8th day includes ten litters, although the frequency distribution includes only two litters, as the others were weighed in groups.

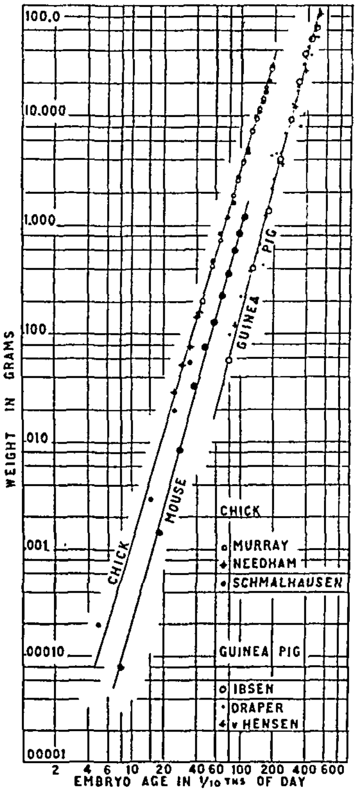
significance has not been discussed. Besides satisfying Brody's exponential function, these data are presented by Robertson (1923)[2] as demonstrating that weight is an autocatalytic function of age. The data are obviously not sufficiently critical to distinguish between these two formulæ. Altogether they include only thirty-eight litters, divided among ten age groups, with ten to forty-four embryos in each group. Some litters were killed as much as 2 hours after the designated 24 hour interval. They were collected over a period of 6 years during which time the diet of the colony was radically changed. With the data being collected at the approximate rate of six litters a year, the question may be raised as to the genetic comparability of the animals used, and also as to the probability of strictly uniform technique in dissection during this long period. These various limitations indicate the desirability of additional data for the rat, and furthermore they provide an explanation of the marked difference between the

FIG. 2. Average weights for embryos of mouse, guinea pig, and chick plotted on logarithmic rulings against embryo age given in units of 1/10th day. Mouse, data of the present authors; sources of other data are indicated on the figure. The straight lines are the graphs of the following equations; velocity constant for the mouse was determined by the method of least squares; for the guinea pig and chick it was obtained graphically.

$$\log W \text{ (mouse)} = 3.649 \log [10\,(t - 7.2)] + \bar{8}.6587$$
$$\log W \text{ (guinea pig)} = 3.987 \log (t - 12) + \bar{5}.1839$$
$$\log W \text{ (chick)} = 3.436 \log [10\,(t - 0.5)] + 7.626.$$

Draper data: each point represents the average of one litter as recalculated from the records for individual embryos in Draper (1920, Table I, pp. 385–386). Ibsen data: each point represents several litters; frequencies of embryos, going up from 8 days embryo age as follows: 17, 15, 23, 16, 24, 18, 17, 17, 54. Von Hensen data: each point is an average of individual embryos from two or more litters, in frequencies as follows: starting with 9 days embryo age: 3, 3, 4, 4, 8, 4, 4. Murray data include the following number of embryos, starting from 4.5 days embryo age: 200, 45, 91, 42, 48, 48, 27, 15, 35, 15, 12, 10, 35, 29, 30. Needham data include the following number of embryos, starting with 2.5 days embryo age: 85, 77, 80, 78, 83. Schmalhausen data include the following number of embryos (frequency of first point not stated): 12, 16, 15, 12, 10, 9, 10, 7, 5, 6, 6, 5, 2, 2, 3, 2, 2. Data beyond minumum prenatal period not included in any case.

[2] Robertson (1923), p. 56.



100.0
10.000
1.000
.100
.010
.001
.00010
.00001
WEIGHT IN GRAMS
2 4 6 10 20 40 60 100 200 400 600
EMBRYO AGE IN 1/10 THS OF DAY
CHICK
MOUSE
GUINEA PIG
CHICK
MURRAY
NEEDHAM
SCHMALHAUSEN
GUINEA PIG
IBSEN
DRAPER
v HENSEN

FIG. 2.

semilogarithmic curves for the rat and the mouse that is more reasonable than the supposition of a real difference in the processes of growth.

When the mouse averages were plotted on logarithmic paper the higher averages approached a straight line, but the lower ones bent markedly downward away from this line. By subtracting different amounts from conception age and replotting each time, it was found that all the data were brought most clearly into a linear relation when 7.2 days were subtracted. The averages for the mouse in Fig. 2 are plotted on this basis. The straight line is the graph of the equation

$$\log W = 3.649 \log [10 (t - 7.2)] + \bar{8}.6587$$

in which W is the weight and t conception age, which is reduced to units of 1/10 day to facilitate the calculations with logarithms.

Our histological evidence agrees with this time relation. Embryos 6 days old show no mesoderm. Of twenty-one 7 day embryos from three litters, sectioned transversely or longitudinally, all but two show mesoderm in varying quantities, sixteen are sufficiently developed for a primitive groove, but none shows a head fold. Of six from a litter of $7\frac{1}{2}$ days, five have reached the primitive groove stage and one has the beginning of the head fold. While there is great variation in our 8 day embryos, very few do not have the head fold and many show somites (up to six). Further embryological data will be published later, but from the evidence here presented it would seem that the primitive streak first appears on the 7th day, and that usually the anlage of the embryo proper is not actively differentiated until the early part of the 8th day. Sobotta (1911) records mesoderm in an embryo at the end of the 7th or the beginning of the 8th day after conception.[3]

This statistical method of determining embryo age has been applied to the data for the guinea pig and the chick.

Guinea Pig.—Probably the most accurate data previously published on prenatal growth of a mammal are those for the guinea pig, Draper (1920), von Hensen (1876), and Ibsen, who has most kindly permitted the use of his averages in advance of their publication. In the guinea pig the extended period of gestation, which is over three times as long

[3] Sobotta (1911), Taf. XIV, Fig. 3.

as that of the mouse and includes the infantile stage that is postpartum in the mouse, gives an excellent test for the applicability of the power function and embryo age as a description of growth *in utero*. Fig. 2 shows that when embryo age is taken as t–12, the logarithms of the average weights plotted against the logarithms of embryo age fall remarkably close to a straight line. This line is defined by the equation

$$\log W = 3.987 \log (t - 12) + 5.1839.$$

As in the mouse, the age modification required to fit a logarithmic straight line very closely approximates the time that elapses before the first differentiation of the embryo proper. Von Hensen (1876) figures 11 day egg cylinders without primitive streaks and 14 day embryos with as many as six somites. Bischoff (1852) states, "Am 13 Tage aber beginnen nun in dem angeschwollenen freien Ende des Eizapfens die merkwürdigsten Veränderungen, die rasch fortschreitend, binnen 48 Stunden den Embryo in seinen meisten Hauptteilen in Dasein rufen." Lieberkühn (1882)[4] presents a surface view of an embryonic disc aged 13 days, which shows the primitive streak with a well developed area of mesoderm. Another drawing[5] of an embryo 13 days 16 hours old shows a primitive streak nearly twice as long and a much larger area of mesoderm.

Given the uniform conditions of uterine life, the same velocity constant describes the growth of the guinea pig from the first organization of the embryo proper through the long range of developmental stages, even including those which correspond to postnatal infancy in many mammals. This suggests that the variations of the curves after parturition in animals with short gestation are not due to the innate constitution of the embryos, but rather to the changed method of life. It is further suggested that the cycles found by Read (1913) at one age and at another by Brody and Ragsdale (1922–23)[6] in the data of Minot, reveal the inaccuracy of their data (weights of pregnant females in both cases) rather than the autocatalytic nature of growth.

Chick.—Murray (1925–26) has shown that the equation $W = .665t^{2.6}$,

[4] Lieberkühn (1882), Fig. 3.
[5] Lieberkühn (1882), Fig. 3*a*.
[6] Brody and Ragsdale (1922–23), Fig. 6, p. 211.

in which t is incubation age, gives an accurate description of his own extensive series of embryo chick weights as well as those of Lamson and Edmond, and Hasselbalch.[7] The weights given by Schmalhausen (1926) also agree with this equation as far down as 5 days, but earlier than this his averages and those of Needham (1927) fall distinctly below the line of Murray's equation. While incubation and embryo age are so very nearly the same that they serve equally well for the larger embryos, the averages for the very early embryos reveal the difference by bending away from the line based on incubation age. All these data are brought into fair agreement with the straight line whose equation is

$$\log W = 3.436 \log [10 (t - 0.5)] + \bar{7}.626$$

in which incubation age t is reduced by half a day and multiplied by 10 to reduce the unit of time to 1/10th day. Fig. 3 shows the averages up to 5 days in comparison with the values given by this formula, by Murray's formula, and by an exponential extrapolation of Murray's averages given by Needham.

Our graphic estimate of embryo age is closely borne out by the embryological data. Duval (1889), whose timing of chick embryos is usually taken as a standard, figures the earliest primitive streak with mesoderm in the neighborhood of the 10th hour of incubation.[8] He shows 16 hour blastodiscs with the primitive streak varying from no primitive pit to the development of a chorda dorsalis. Jenkinson (1913)[9] states, "—and soon the first sign of the embryo appears (about the 12th hour of incubation) in the form of the primitive streak, ..." This is shown in his Fig. 105*a*.

Other descriptions of the weight-age function for chick embryos have appeared. Schmalhausen shows[10] that the cube roots of weight plotted against age roughly approach a straight line. This is the same power function used by Murray but expressed in a different way with a less accurate velocity constant. In this same figure Schmalhausen demonstrates that the logarithms of weight against age form a

[7] Murray, Fig. 5, p. 45.
[8] Duval (1889), Plate III, Figs. 47 and 62.
[9] Jenkinson (1913), p. 172.
[10] Schmalhausen (1926), Fig. 1, p. 339.

smoothly graded curved line. Brody (1926–27) draws a series of straight lines through corresponding exponential curves for different series of chick data, including those of Murray, and concludes that

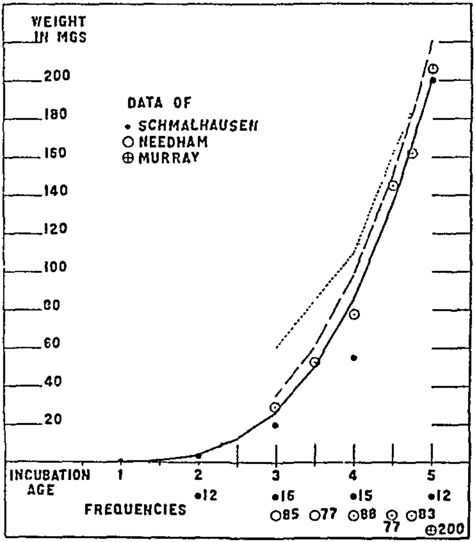


FIG. 3. Average weight for chick embryos, 1 to 5 days incubation age. Solid line is the graph of the equation based on embryo age given, under Fig. 2, for the chick; broken line is the graph of the formula given by Murray. The dotted line is from figures given by Needham (1927, p. 261, Table I, Column 5) as an extrapolation of Murray's averages based on the assumption that embryos grow at the same rate before 5 days as between 5 to 7 days,—that is, an exponential function. (Needham's Fig. 1 gives a curve which does not entirely agree with the numbers given in his table.)

growth rate does not decline continuously, but by abrupt drops between periods of uniform rate. Since any curve can be approximated by a series of straight lines, the critical significance, both of the specific

number of straight lines used and of his general conclusions, seems somewhat questionable.

CONCLUSIONS.

1. The general course of prenatal growth in the mouse, the guinea pig, and the chick can be expressed by straight line relations between the logarithms of the weight and age only when age is counted from the beginning of the embryo proper.

2. This is interpreted as showing that the manner of growth before the beginning of the embryo proper is essentially different from that after this time.

3. The velocity constants for the animals mentioned are similar; the major differences in their curves depend on the amount of tissue involved in the first organization of the embryo proper and in the length of prenatal life.

4. Growth of different animals may be compared more accurately if, instead of either birth age or conception age, embryo age is used.

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ON THE TITRATION OF BACTERIOPHAGE AND THE PARTICULATE HYPOTHESIS.

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INTRODUCTION.

One of the methods most frequently used to estimate the concentration of bacteriophage in a fluid medium is that of serial dilution. If several parallel titrations of the same solution are made by this method, it will be found usually that the results are not entirely consistent; that, although in most cases the number of tubes in which the bacteria dissolve will be the same, let us say n, a few cases will yield $n \pm 1$.

Dr. Bronfenbrenner,[1] of The Rockefeller Institute, in whose laboratory many thousands of such titrations have been made on solutions of various degrees of concentration, estimates that, if the dilution factor be .1, about 85 per cent of such parallel runs yield the same value of n. This degree of consistency is about 40 per cent higher than one would expect if it is true, as is quite generally believed, that bacteriophage exists in the state of particles, a single one of which is sufficient when added to a culture of susceptible bacteria to start the destructive processes.

Dr. Bronfenbrenner's estimate is based largely on the general impressions gained by himself and his coworkers in the course of much experimental work rather than on definite records. The discrepancy between this estimate and the results of analysis is so great, however, that it deserves consideration. It should be checked by experiment. If the predictions of theory are upheld, it would constitute an interesting verification of the simple particulate hypothesis. If not, it would require a further consideration of the hypotheses on which the analysis

[1] I am indebted to Dr. Bronfenbrenner for his kindness in furnishing the material on which this paper is based.

is based, which in itself might prove to be of interest. Inasmuch as the labor involved in making the experiments is very great, such a check can best be made as a by-product of titrations made for other purposes. A brief presentation of the analysis together with a discussion of the hypotheses on which it is based, may, therefore, be of interest.

The Serial Dilution Method.

The method will be explained briefly by an example. We put 10 cc. of the solution to be titrated, which contains broth as well as bacteriophage, into the first of a series of test-tubes; into each of the other tubes, we put 9 cc. of sterile broth. We now remove 1 cc. of the fluid from the first tube and introduce it into the second. After very thorough stirring, we remove 1 cc. from the second tube, using a clean pipette, and put it into the third tube. We continue this process indefinitely, in so far as the theory is concerned. The expectation of bacteriophage in any tube is, therefore, exactly one-tenth as great as that for the preceding tube. The quantity, .1, is called the dilution factor. Susceptible bacteria are now put into each of the tubes. In the first n tubes, they dissolve; in all of the others, they live and multiply. 10^{n-1} is taken as a measure of the concentration of the original solution.

Statistical Treatment of the Problem.

It will be assumed for the present that the presence of one or more particles of bacteriophage in any tube always results in the dissolution of all of the bacteria, that particles neither dissociate nor coalesce during the process of dilution, and that none of the particles are lost by adsorption or otherwise. The effects of changing these hypotheses in various ways will be discussed later.

Let x = the exact number of particles of bacteriophage placed in the first tube,

p_n = the probability that the last (most dilute) tube in which the bacteria dissolve will be the nth tube of the series, and

a = the dilution factor.

In what follows, it will be assumed that $a = .1$ unless otherwise stated.

The probability that the $(n+1)$th tube receives *a particular one* of the particles originally in the first tube is a^n; the probability that it does *not* receive it is $1 - a^n$; and the probability that it receives *none*

of the original x particles is, therefore, $(1-a^n)^x$. Likewise, the probability that the nth tube receives none is $(1-a^{n-1})^x$. These probabilities are not independent, however; whenever the nth tube receives none the $(n+1)$th tube *must* also receive none. In every other case in which the $(n+1)$th received none, the nth must have received *some*, and it must have *retained* them. Therefore, the probability, which is in effect p_n, that the nth retains at least one and the $(n+1)$th receives none is given by

$$p_n = (1-a^n)^x - (1-a^{n-1})^x \tag{1}$$

If x and n are infinite, xa^n being finite, this equation may be written

$$p_n = e^{-xa^n} - e^{-xa^{n-1}} \tag{2}$$

These are the fundamental equations with which we shall have to deal in what follows.

The Maximum Value of p_n for Small Values of n.

Let P_n = this maximum value of p_n, and
X_n = the value of x which corresponds to P_n.

If $n=1$, it is obvious that $X_1 = 1$. Tube 1 must retain at least one particle, and the smaller the number of particles it receives, the less the probability that it will lose one of them to Tube 2. Equation (1) shows, then, that $P_1 = 1 - a$ which is .9.

If $n > 1$, we can find between what two consecutive integral values of x the desired value lies by treating x as a continuous variable. Accordingly, we set D_xP_n equal to zero. From equation (1), we find that

$$D_xP_n = (1-a^n)^x \log_e (1-a^n) - (1-a^{n-1})^x \log_e (1-a^{n-1}).$$

Setting this expression equal to zero, simplifying, and writing X_n in place of x, we have

$$X_n = \frac{\log[-\log(1-a^{n-1})] - \log[-\log(1-a^n)]}{\log(1-a^n) - \log(1-a^{n-1})} \tag{3}$$

in which the base of logarithms is arbitrary.

Column 2 of Table I contains the values of X_n found by setting a equal to .1, and n equal to 2, 3, and 4 in equation (3), and Column 3 contains the corresponding values of P_n found by substituting X_n in equation (1). These quantities cannot be less than the true values corresponding to the best integral values of X_n. Inspection of Column 3 shows that as n increases from 1 to 4, P_n apparently approaches a limiting value very rapidly. To make sure of this, we must find the value of P_n when n is infinite.

TABLE I.

1	2	3	4	5	6	7	8
$a = .1$						$a = .09$	
n	X_n	P_n	X'_n	p'_n	$\bar{p}_n$	P_n	p'
1	1.000	.900	7.27	.466		.910	.469
2	24.60	.706	76.6	.463	.604	.720	.467
3	255.0	.698	770.	.463	.602		
4	2558.	.697					
∞		.697		.463	.602	.717	.467

X_n is the value of x corresponding to P_n, the maximum value of p_n which in turn is the probability that the last (most dilute) tube in which bacteria dissolve is the nth tube of the series.

X'_n is the value of x for which $p_n = p_{n+1}$. At this point, p'_n, the degree of consistency of parallel runs, has a minimum value.

$\bar{p}_n$ is the mean value of p_n over the range of values within which p_n is greater than p with any other subscript.

The Value of P_n When n Is Infinite.

From equation (2), we find that

$$D_x P_n = a^{n-1} e^{-xa^{n-1}} - a^n e^{-xa^n}$$

setting this expression equal to zero, simplifying, and writing X_n for x, we find

$$X_n = \frac{-\log_e a}{a^{n-1}(1-a)}$$

After substituting this expression for x in equation (2), and simplifying, it appears that

$$P_\infty = a^{\frac{a}{1-a}} - a^{\frac{1}{1-a}} \tag{4}$$

The value of P_∞ given in Column 3 was found by setting a equal to .1 in equation (4).

The Value of p'_n for Small Values of n.

Between X_n and X_{n+1}, there must be a value of x for which $p_n = p_{n+1}$. We denote this value by X'_n. When $x = X'_n$, the degree of consistency of titrations of samples containing the same number of particles will have a minimum value inasmuch as it is equally probable that a run will yield either n or $n+1$. The value of p_n corresponding to X'_n will be denoted by p'_n. We proceed to find the values of X'_n. Setting the expression given by equation (1) for p_n equal to a similar expression for p_{n+1}, rearranging terms, and writing X'_n for x, we have

$$\left[\frac{1-a^n}{1-a^{n-1}}\right]^{X'_n}\left\{2-\left(\frac{1-a^{n+1}}{1-a^n}\right)^{X'_n}\right\} = 1 \tag{5}$$

Equation (5) shows that $X'_1 = 7.27$. For higher values of n, the equation cannot be solved for X'_n explicitly, but the values of X'_n can be found to any desired degree of approximation as follows: We set the quantity inside the brackets equal to zero, thus—

$$X'_n = \frac{\log 2}{\log(1-a^{n+1}) - \log(1-a^n)} \tag{6}$$

Having found a value of X'_n for any small value of n from equation (6), we use this value as the exponent of the first parenthesis of equation (5). This gives a new value of the quantity inside the brackets slightly different from zero, and consequently a new equation like equation (6) except that the figure 2 is replaced by a quantity slightly less than 2. This process could be carried on indefinitely but inspection shows that the true final value of X'_n cannot differ from the value first found from equation (6) by as much as .1 of 1 per cent for any value of n. We, therefore, use equation (6) and ignore the error in-

volved. Column 4 of Table I contains the values of X'_n thus found and Column 5 contains the corresponding values of p'_n found by substituting the values in Column 4 together with the corresponding values of n in equation (1). Inasmuch as X'_n must, in fact, be an integer, these values are slightly too small. The error is certainly negligible if n is 2 or more.

The Value of p'_n When n Is Infinite.

To make sure that p'_n approaches a limiting value as n increases, we find the value of p'_n when n is infinite. As in the preceding section, we first set p_n equal to p_{n+1} to find X'_n. Using equation (2) for the purpose, writing X'_n for x, and introducing a new variable, y, such that

$$X'_n = \frac{\log_e y}{a^{n-1}(1-a)}, \tag{7}$$

we find that

$$y(2 - y^a) = 1 \tag{8}$$

Equation (8) is the analogue of equation (5), and the value of y can be found by the same method of approximation. Inspection shows, as before, that

$$y = 2^{\frac{1}{a}} \tag{9}$$

gives log y with a maximum error of .1 of 1 per cent. Substituting this value of y in equation (7), we have

$$X'_n = \frac{\log_e 2}{a^n(1-a)} \tag{10}$$

and this expression when substituted in equation (2) gives

$$p'_\infty = 2^{\frac{1}{a-1}} - 2^{\frac{1}{a(a-1)}} \tag{11}$$

p'_∞ is, therefore, equal to .463 with an error of less than .1 of 1 per cent.

The Value of $\bar{p}_n$ for Small Values of n.

If we are working with solutions of a great variety of degrees of concentration, we are justified in considering the mean value of p_n for the values of x which lie between X'_{n-1} and X'_n. This quantity is denoted by $\bar{p}_n$. If n is greater than 1, we may treat x as a continuous variable without introducing an appreciable error. We simply integrate $p_n dx$ (using equation (1) for the purpose) between the limits X'_{n-1} and X'_n, and divide by the difference of the limits. We find, thus, that

$$\bar{p}_n = \frac{1}{X'_n - X'_{n-1}} \left[\frac{(1-a^n)^{X'_n} - (1-a^n)^{X'_{n-1}}}{\log_e (1-a^n)} - \frac{(1-a^{n-1})^{X'_n} - (1-a^{n-1})^{X'_{n-1}}}{\log_e (1-a^{n-1})} \right] \tag{12}$$

The values of $\bar{p}_n$ for $n = 2$ and $n = 3$ shown in Column 6 were found by substituting the figures of Column 4 together with the appropriate values of n in equation (12).

The Value of $\bar{p}_n$ When n Is Infinite.

As before, we integrate $p_n dx$ from X'_{n-1} to X'_n (using equation (2)) and divide by the difference of the limits. X'_n is given by equation (10) and X'_{n-1} is a similar expression with the value of n reduced by one unit. After integrating, substituting these expressions for the limits, and simplifying, it comes out that

$$\bar{p}_\infty = \frac{1}{\log_e 2} \left[2^{\frac{a}{a-1}} + a \cdot 2^{\frac{1}{a(a-1)}} - (1+a)\, 2^{\frac{1}{a-1}} \right] \tag{13}$$

which proves to be .602.

The Effect of Altering the Dilution Factor.

In the preceding pages, a has been taken as .1. Increasing the value of a would result in a lowering of the values of the various p's; and conversely a decrease in the value of a would have the opposite

effect. To make sure that a slight change in the dilution factor could not produce a great change in the results, I have recalculated P_n and p'_n taking .09 for a. The results are shown in Columns 7 and 8. If we set a equal to zero in equation (13), we find that the limiting value of $\bar{p}_\infty$ is .722.

DISCUSSION.

In the foregoing, it has been necessary to deal with x as a continuous variable and to consider the case in which x is infinite. One must be careful not to confuse these analytical devices with the idea that the active substance is itself infinitely divisible; they were used simply for the purpose of studying equations (1) and (2) which are based on the particulate hypothesis. The low values of the p's in Table I are brought about by the fact that, however nicely the active substance may be divided by serial dilution in the first stages where the number of particles per cc. is great, a time comes as the dilution continues when the number of particles per cc. is so small that the probability variations are considerable. It is by the indications at this point that the state of affairs in the first tube is judged.

In practice, n is much greater usually than 1 or 2. We may, therefore, ignore these two cases. We take a as .1. Table I shows that a value 10 per cent lower makes little change in the results; we may, therefore, ignore the effects of slight errors of dilution.

The table shows that if $n>2$ all of the p's are practically independent of n. It makes little difference, then, whether a particular tube, (the first tube as we have taken it) receives exactly x particles, whether it is made from a parent tube the concentration in which is ten times as great, or whether it is merely a sample of stock solution.

It appears from Column 3 that, with a fortunate choice of the solution to be titrated, about 70 per cent of parallel runs might yield the same value of n. On the other hand, if the choice were unfortunate, less than half of them would yield the same value of n. In the long run, working with a great variety of solutions, we should expect 60 per cent to yield the same value. The discrepancy between this figure and Dr. Bronfenbrenner's estimate, 85 per cent, based on the actual yield of the method in practice is, in Dr. Bronfenbrenner's opinion, too great to be ignored.

It will be remembered that our analysis of the problem was based on the simple assumption that only one particle need be put into a tube in order to dissolve the bacteria in it. It has not been assumed that the particles are alike. The particles may be molecules—all alike—or they may consist of particles of foreign matter on the surfaces of which one or more of the ultimate units of bacteriophage have been adsorbed. We have required only that particles neither divide nor coalesce during the process of dilution (only the second of these processes would make $\bar{p}_n$ greater). It is, of course, conceivable that, in concentrated solution, a change of concentration might have some influence on such particles, but it is hard to imagine how any such change could take place during the process of serial dilution after a point has been reached where there are only from one to ten particles in 10 cc. of broth. Such changes in the first part of the series would have a profound effect on the accuracy of estimates made by the method, but none on the degree of consistency of the results.

It is conceivable that the interaction of a bacterium and a particle of bacteriophage is, in itself, a matter of probability. The particle may be inactive, or it may attach itself to a bacterium which is not susceptible. It is reasonable to assume that, of the whole number of bacteria added to each tube, a constant fraction are susceptible. We may say, then, that there is a certain constant probability, q, that any particular unit of bacteriophage will act effectively. This could have been taken into account very easily in deriving equations (1) and (2), thus—if, instead of considering the probabilities, a^{n-1} and a^n, that a particular unit of bacteriophage would be transferred to the nth and $(n+1)$th tubes respectively, we had considered the probabilities that the particular unit would act effectively in these two tubes, we should have found them to be $a^{n-1}q$ and a^nq respectively. q may now be replaced by some unknown positive power of a. It is evident, therefore, that the effect of introducing q is to increase the value of n. This means that the limiting values of the p's remain unchanged and that the values of the p's for small values of n, are, for the same value of n, more nearly in coincidence with the limiting values than they would be if q were not introduced; in other words, if n is greater than 2, the introduction of q is without appreciable effect.

We have next to consider adsorption losses. During the process of

stirring and transferring fluid, some of the particles must come in contact with the surfaces of the tube and the pipette and it may be that some or all of them adhere to the glass. Although this would not necessarily render the particles inactive, it would effectively prevent transferring them to the next tube. Such losses, if they exist, must be very small. Dr. Bronfenbrenner[1] has found that a very dilute solution (corresponding approximately to Tube $n - 2$) gives the same value of n whether it is titrated immediately after preparation or after having been kept in glass for 72 hours. This means that, during the 10 minutes required to make a transfer, only a very small fraction, certainly much less than 10 per cent, of the whole number of particles in the tube will be adsorbed. Since the fraction is so small, and since the transfers to successive tubes require about the same length of time, we may say that there is a definite probability, which is the same for all of the transfers, that any particular unit of bacteriophage, which has been transferred to any tube, will escape adsorption until the transfer of fluid to the next tube has been made. This probability may be combined with the dilution factor, a, to give a new and slightly smaller value of a. If 10 per cent of the particles were lost at each transfer, a would be reduced from .1 to .09. Table I shows that the corresponding increases in the values of the p's amount to only 2 or 3 per cent.

If, therefore, it is true that when one active particle of bacteriophage comes in contact with a susceptible bacterium, all of the bacteria in the tube dissolve, we are justified in expecting that, in the long run, about 60 per cent of parallel runs will yield the same value of n. This figure will remain unaltered whatever value we assign to the probability either that a particle is by nature inactive, or that it is taken up by a bacterium which is not susceptible; and it will change only slightly as a result of the greatest adsorption losses which we have reason to consider.

If experiment should show definitely that the serial dilution method yields results with a degree of consistency much greater than 60 per cent, the most obvious explanation of the discrepancy will be that one particle is not usually sufficient to cause the dissolution of all of the bacteria in the tube, even though it is active and comes in contact with a susceptible bacterium. This idea is not seriously in conflict

with the most important feature of the particulate hypothesis as usually understood, *i.e.* that one particle can start the process of dissolution. It is conceivable that a single infected bacterium may not be able to produce enough particles of bacteriophage to infect all of the others within the time during which the bacteria remain susceptible.

SUMMARY.

1. The theory of the serial dilution method of titration of bacteriophage has been worked out on the basis of the simple particulate hypothesis.

2. It has been shown that, if the dilution constant is .1, only about 60 per cent of parallel runs on the same solution should give the same end-point, the average being taken over a great number of titrations of each of a great variety of solutions.

3. The discrepancy between this figure, 60 per cent, and Dr. Bronfenbrenner's estimate, 85 per cent, is considerable.

4. Inasmuch as the particulate hypothesis is well founded, no explanation of the discrepancy is suggested.

SOME ASPECTS OF BIOELECTRICAL PHENOMENA.*

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This brief sketch is intended as an introduction to a series of articles[1] on bioelectrical phenomena, its purpose being to present certain fundamental facts and underlying conceptions.

Early in the course of the investigation it became evident that there are great advantages in using single cells in place of tissues. The experiments were accordingly made with single (multinucleate) cells of *Valonia* and *Nitella*, which are large enough[2] to permit leading off simultaneously from several places on the same cell. This has important technical advantages and eliminates certain complications[3] which always arise in the study of tissues. In addition it enables us to find out to what extent changes in any part in the cell may affect other parts. A study of such effects and of their transmission in protoplasm may be expected to throw some light on the propagation of stimuli in general and on the constitution of living matter.

Another advantage attending the use of these cells may be mentioned here. The study of bioelectrical phenomena has been hampered because nothing could be measured except potential differences between selected spots, and it has been impossible to determine the

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[1] The author desires to express his gratitude to the Carnegie Institution of Washington, D. C., which made possible the beginning of these investigations.

[2] The cells of the marine alga *Valonia* reach a length of 2 inches or more and those of the fresh water *Nitella* a length of 5 inches or more. The cells consist of a thin layer of protoplasm (containing numerous chloroplasts and nuclei) outside of which lies the cell wall and inside of which is the very large central vacuole filled with cell sap.

[3] For example, in a tissue the circuit includes a number of cells between which is intercellular material of some sort. If a cell is injured cell sap comes out and alters the intercellular material which in turn alters the potential difference of the uninjured cells. With single cells this cannot occur.

absolute value of the potential difference across the protoplasm at any one point. Since it is highly desirable to obtain such absolute values an attempt was made to do so. It would not be possible to make such determinations in tissues or in cells of ordinary size, but the use of very large cells enables us to reach the desired end.

In the case of *Valonia* this was done by piercing the cell with a capillary glass tube filled with cell sap (Fig. 1). On leading off from the interior of this tube to the outside of the cell we obtain a circuit which passes only once through the protoplasm (as indicated by the dotted line), and hence the measured E.M.F. gives the potential difference across the protoplasm at any point where an external contact is applied. In many cases the protoplasm attaches itself to the capillary at *F* so as to form an electrical seal, thus preventing any short circuit through the wall (between *F* and *G*) and along the outside of the capillary into the sap, and only such cells were employed in the experiments.

In the case of *Nitella* the same purpose was accomplished by reducing the potential difference at one point approximately to zero, by killing the protoplasm in such fashion[4] as not to affect other points some distance away on the same cell (at least for some time). In leading off from the killed point to a normal region the circuit passed once through the killed spot and once through living protoplasm, and the results justify the conclusion that when the experiments are made under the proper conditions the observed electromotive force is practically all due to the potential difference across the living protoplasm at the selected point.

In order to interpret the results of our measurements we need information regarding the structure of the protoplasm. There is some evidence to show that in general the surface of protoplasm differs from its interior, and some experiments indicate that the surface is non-aqueous. The interior of the protoplasm may be an aqueous phase consisting of sol or gel or both, or it may be an emulsion in which the outer phase is aqueous. We might therefore, as a working hypothesis, consider the protoplasm to be made up of an aqueous phase, *W*, and phases which are probably non-aqueous, forming the

[4] This can be done in a variety of ways which will be discussed in detail in subsequent papers.

external (X) and internal (Y) surface. These layers may be very thin (possibly monomolecular), or if thicker they may consist of sol or of gel or of an emulsion, the outer phase of which is non-aqueous.

It should be emphasized that this conception is set up merely as a working hypothesis which may be useful for the time being (some new evidence for the existence of layers will be presented in later papers). It is quite possible that the boundary surfaces are aqueous in character, and if the protoplasm really consists of layers it is quite possible that there are more than three. For the present, however, we shall adhere to the hypothesis in the form presented above.

Let us now consider under what conditions bioelectric effects may be expected to arise. If the protoplasm is made up of layers it may, for convenience, be represented as in Fig. 1 (in which G represents a salt solution applied to the cell wall and quickly penetrating through it to the surface of the protoplasm). We shall discuss certain possibilities on the assumption that these layers exist. It will then be evident what conditions would obtain if the protoplasm were not made up of layers.

Let us first consider the cell wall. This is of cellulose, and the experiments show that it is readily permeable to salts; so that an applied salt solution quickly penetrates the cell wall and comes in contact with the external surface of the protoplasm. If the salt solution has ions which move at different rates in the cell wall a diffusion potential will be set up. This however would not last long if the salt diffused only at right angles to the surface since the cell wall is very thin and very permeable; but a potential difference due to diffusion along the wall (from G toward F) might last a long time, but this would have little or no effect on the E.M.F. in the cell, measured as shown in Fig. 1, since in the experiments only those cells were used in which the protoplasm had made an electrical seal[5] at F so that no current

[5] It is an easy matter to tell whether this seal is made. If we place 0.6 M KCl at G and lead off from G to a drop of 0.6 M KCl placed on the outside of the cell at F (*i.e.* at the point where the capillary enters) it is evident that if there is a leak around the capillary we shall get the same potential difference as if we led off from G to the interior of the capillary; when the seal is made we actually observe a very different value. We arrive at the same result if we first lead off as shown in Fig. 1 and then immerse the cell completely in the solution applied at G. This will be discussed in later papers.

could leak along the outside of the capillary; hence the wall did not form a short circuit between *F* and *G* and any potential difference due to the diffusion of solution in the wall from *G* toward *F* would probably have only a negligible effect on the measured E.M.F.

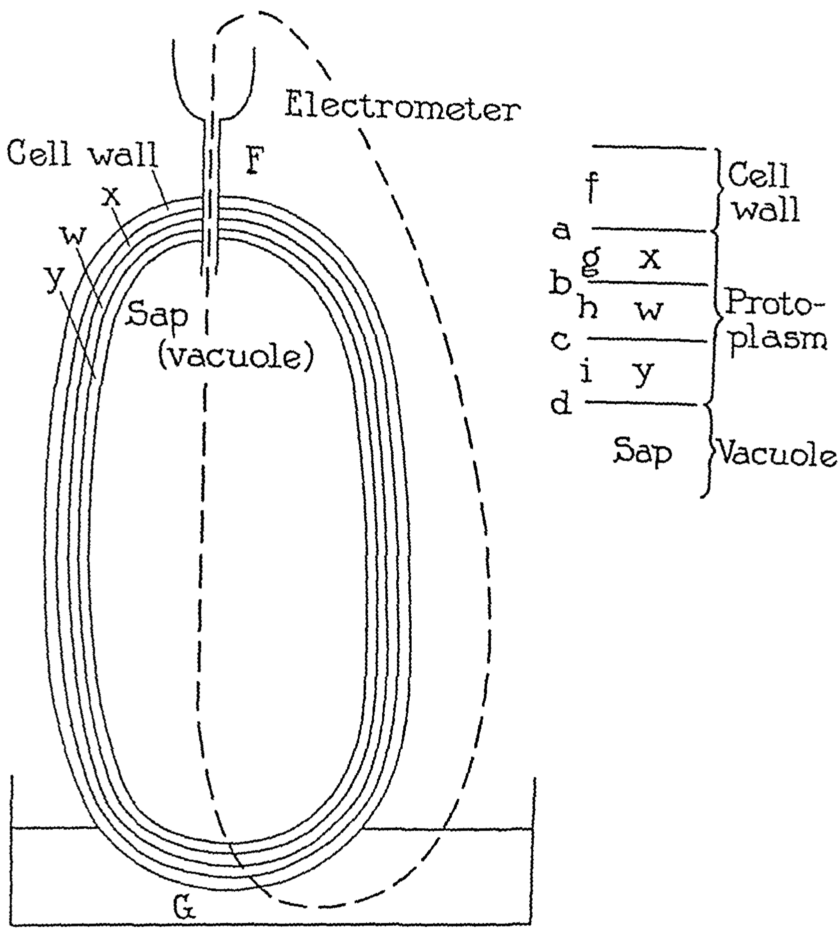

FIG. 1. Diagram of a cell of *Valonia* with inserted capillary. The thickness of the protoplasm and cell wall is exaggerated, being only a few microns, while that of the vacuole may be over an inch: *a*, *b*, *c*, and *d* are the seats of phase boundary potentials, and *f*, *g*, *h*, and *i* the seats of diffusion potentials. The circuit is supposed to follow the course of the dotted line.

What has been said about diffusion in the cell wall might apply to any of the other layers which are readily permeable to salts; but *X* and *Y* may be nearly or quite impermeable.

In addition to diffusion potentials we may consider phase boundary potentials, which may arise for example at *a*, *b*, *c*, and *d* (providing *X*, *W*, and *Y* represent distinct phases). The cell wall is here omitted from consideration since it appears too permeable to be the seat of phase boundary potentials.

The outer layer *X* is probably permeable to some extent to certain ions, which may give rise to potential differences when brought in contact with it: in this case current[6] must be able to pass through *X*.[7]

We must consider the possibility that *Y* may be almost or quite impermeable to ions, a possibility which is indicated by the situation in *Valonia*. Little or no Mg or SO_4 penetrates the vacuole, yet it seems probable that the protoplasm contains S and the chlorophyll bodies embedded in the protoplasm must contain Mg. It might therefore seem possible that Mg^{++} and SO_4^{--} penetrate *X* but not *Y* (unless $MgSO_4$ penetrates *X* only in the form of undissociated molecules). (It is also possible that the continuity of *X* is interrupted over each chlorophyll body so that Mg can gain access to it without passing through *X*.) If the layer *Y* is impermeable to ions generally, it is evident that the potential at both its surfaces, *i.e.* at *c* and *d* (Fig. 2), might under certain conditions be zero.

If *Y* were almost or quite impermeable to ions this would explain certain facts[8] which indicate that in general ions cannot penetrate readily into the vacuole. If we suppose that all the layers are permeable to ions we should assume that under normal con-

[6] The mere fact that E.M.F. produced at *B* and *C* can affect the measuring instrument does not prove that *X* conducts much current, since a very minute current can keep the electrometer charged, as can be shown by inserting a condenser in series with the cell.

[7] If *X* were aqueous it would of course conduct.

[8] This evidence has been gathered chiefly from studies by a number of investigators on the penetration of weak acids (for references see Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–27, viii, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255) and of bases, as well as of dyes (*cf.* Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561), which show that ions penetrate very slowly or not at all. The experiments of several investigators, especially unpublished results of Dr. Blinks, show that the resistance of the protoplasm is very high and unless this is due to polarization it must indicate a very low degree of permeability to ions on the part of some or all of the layers.

ditions this permeability is very slight. What has previously been said regarding the protoplasm applies especially to the marine alga *Valonia macrophysa*. Let us now consider the situation in the fresh water plant *Nitella*. In this case it is difficult to insert a capillary on account of the small size and the delicacy of the cells. We therefore perform the experiments by leading off from two places, as at *B* and *C*, Fig. 2. For convenience we shall postulate during the present discussion that the current flows chiefly in the circuit indicated by the dotted line. There may, however, be a short circuit in any layer. It seems probable that the only layer in which such

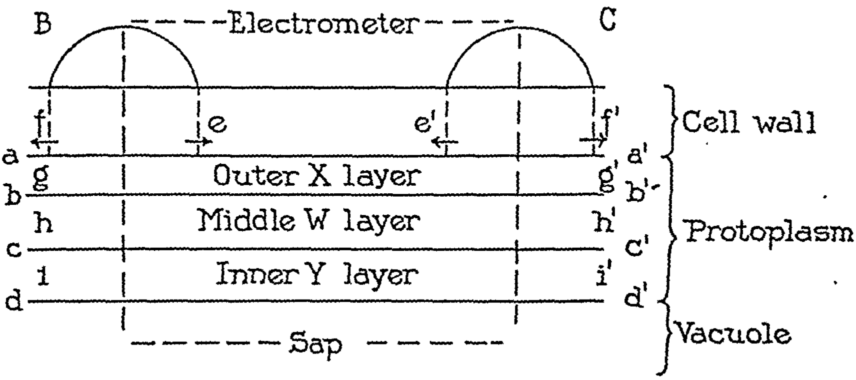

FIG. 2. Hypothetical diagram of a portion of a cell. *B* and *C* represent drops of solution applied to the exterior: *a*, *b*, *c*, and *d* represent the surfaces of the layers and are the seats of phase boundary potentials; *e*, *f*, *g*, *h*, and *i* represent the seats of diffusion potentials in the cell wall and in the layers of protoplasm. The main circuit is supposed to follow the course of the dotted line. The thickness of the cell wall and of the protoplasm is only a few microns.

short circuiting is important is the cell wall (the other layers being probably too thin or too resistant to permit much current to flow), and even in the cell wall this effect is apparently very small when it is imbibed with distilled water[9] or with tap water, as in *Nitella*. In *Valonia*, where the cell wall is imbibed with sea water, the short-circuiting effect would become important if the protoplasm did not attach itself to the capillary to form an electric seal (at *F*, Fig. 1)

[9] But the diffusion potentials in the cell wall due to the solutions at *B* and *C* might be greater in some cases when the wall was imbibed with distilled water than when imbibed with a salt solution.

which prevents short circuiting; this seal was secured in all the experiments.

If the solution applied at B differs from the solution with which the cell wall is imbibed, potentials may arise at e and f, which may set up "eddy" currents[10] flowing through the cell wall and through the protoplasm back to the drop applied at B. Their magnitude would depend on the potential differences as well as on the resistances involved. It is difficult to say what effect they would have upon the current which follows the course indicated by the dotted line in Fig. 2, but it seems probable that any effect will be of brief duration.[11] Similar "eddy" currents might be set up in any of the layers.

When identical solutions are placed on B and C it frequently happens that little or no potential difference is observed. Under these circumstances it seems reasonable to assume that the potential difference at a is equal and opposite to that at a', etc., and that diffusion potentials likewise cancel out. If the solutions applied to B and C in such cells are not identical it is probable that all the values except those at a and a' and at g and g' are equal and opposite. This would, of course, differ from the circuit in *Valonia*, as shown in Fig. 1. In other respects, however, what is said of *Valonia* applies to *Nitella*, and the following discussion applies to both.

Solutions applied to the surface will probably not affect the deeper layers for some time (unless they are very toxic solutions which break down or alter X), so that in brief experiments with non-toxic solutions we may consider that any observed changes depend only on the effect upon X and it will make no difference in the interpretation of the results whether we regard the protoplasm as consisting of one or of many layers. The hypothesis that there are several layers becomes important when we deal with toxic effects or other alterations in the protoplasm.

Polarization may, of course, be expected at any of the layers with a consequent diminution of the current.

The observed potential difference may be made up of the phase

[10] There is, of course, a current flowing from B to C through the cell wall and back through the galvanometer, as already mentioned.

[11] The experiments show that in general when one solution is substituted for another the observed changes are completed in a few seconds unless injury occurs.

boundary potentials at *a*, *b*, *c*, and *d*, and of the diffusion potei in *X*, *W*, and *Y*. In addition there may be a diffusion potenti the cell wall, but this will be of short duration if it is due sole diffusion across the wall, since the wall is very permeable: if due to diffusion along the wall it may last for some time.

A potential difference is usually observed when we lead off fro and C[12] (Fig. 2) with solutions of the same salt at different cor trations (concentration effect), or with solutions of different (chemical effect). In general we observe both effects with pı plasm but the experiments show that in the cell wall with the solut thus far employed only the concentration effect is of importa It is possible to arrange the experiments in such fashion that the e due to the protoplasm can be ascertained, at least approximat Throughout this paper the effects discussed are those on the pr plasm unless otherwise stated.

It is commonly observed that when a solution of KCl is applie one point and a solution of NaCl of the same molar concentra at another point, KCl is negative to NaCl. How is this to explained?

If we regard the whole effect as due to diffusion potential we r say that the mobility of K in the outer protoplasmic layer[13] (*X*,] 1) is greater than that of Na. This is to be expected if the laye behaves, for example, like phenol, as described by Nernst and Ries feld,[14] or like the collodion membranes studied by Michaelis a Perlzweig.[15]

The fact that a concentrated solution of KCl is negative to a dil solution would mean that K penetrates more rapidly than Cl. T would leave the solution negatively charged, the effect being grea

[12] The corresponding experiment is performed with *Valonia*, as in Fig. by leading off from *G*, first with one solution, then with the other, and taking difference between the two measurements.

[13] In the brief experiments here referred to it is not probable that any of deeper layers are involved since the potential differences with which we are h dealing are established within a few seconds. The cell wall appears to play lit or no rôle in connection with the chemical effect.

[14] Nernst, W., and Riesenfeld, E. H., *Ann. Physik*, 1902, viii, series 4, 6

[15] *Cf.* Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926–27, x, 5 where references to earlier papers are given.

as the concentration increases: hence the concentrated solution would be negative to the dilute solution. Since in general in biological experiments dilute solutions of salts are usually positive to more concentrated solutions of the same salt, we might conclude that in general cations tend to penetrate more rapidly than anions (this interpretation would not necessarily hold if the potential differences were due to phase boundary potentials).

Let us now consider phase boundary potentials. The foundation of the theory of these potentials was laid by Nernst.[16] He assumes that the tendency to enter is not the same for all ions. Thus, let us suppose that we have to do with LiCl, and that the concentration of Li in the external solution is C_{Li} and that it tends to enter X and to reach the concentration $A_{Li}C_{Li}$ in X, A_{Li} being the "true" partition coefficient[17] of Li. The corresponding coefficient of Cl is A_{Cl}, and if this is less than A_{Li} (*i.e.* if Cl is less soluble in X than Li is) Li will be unable to reach its "true" value, since it cannot enter in excess of Cl (except perhaps at the very surface), but Cl will enter in excess of its "true" value. The actual concentrations reached in X may be called C'_{Li} and C'_{Cl} and these must be equal. Nernst shows that this leads to the equation

$$\text{P.D.} = RT \log \frac{C_{Li}A_{Li}}{C'_{Li}} = -RT \log \frac{C_{Cl}A_{Cl}}{C'_{Cl}}.$$

Hence

$$\log \frac{C_{Li}A_{Li}}{C'_{Li}} = -\log \frac{C_{Cl}A_{Cl}}{C'_{Cl}} \text{ and } \frac{C_{Li}A_{Li}}{C'_{Li}} = \frac{C'_{Cl}}{C_{Cl}A_{Cl}}.$$

Multiplying both sides by $\dfrac{C_{Li}A_{Li}}{C'_{Li}}$ and substituting the values

$$C_{Cl} = C_{Li} \text{ and } C'_{Li} = C'_{Cl}, \text{ we obtain } \frac{C_{Li}A_{Li}}{C'_{Li}} = \sqrt{\frac{A_{Li}}{A_{Cl}}}.$$

[16] Nernst, W., *Z. physik. Chem.*, 1892, ix, 140; Nernst, W., and Riesenfeld, E. H., *Ann. Physik*, 1902, viii, series 4, 600. *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i.

[17] The "true" partition coefficient is that which would be observed if Li could enter unhindered by Cl: this would be the case if the "true" partition coefficients of Li and Cl were equal.

Hence

$$\text{P.D.} = RT \log \sqrt{\frac{A_{\text{Li}}}{A_{\text{Cl}}}} = \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Cl}}}.$$

Haber[18] arrives by a different route at a formula which reduces to the same thing. Haber's formula is

$$\text{P.D.} = RT \log \left(\frac{C_{\text{Li}}}{C'_{\text{Li}}}\right) (K_{\text{Li}})$$

where K_{Li} is the solution tension of an imaginary Li electrode in X divided by its solution tension in water. It is evident[19] that K_{Li} is equal to the A_{Li} of Nernst's formula.

Hence we may write

$$\text{P.D.} = \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Cl}}} = \frac{RT}{2} \log \frac{K_{\text{Li}}}{K_{\text{Cl}}}.$$

If we apply LiCl at one point and NaCl at another the E.M.F. will be

$$\text{P.D.} = \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Cl}}} - \frac{RT}{2} \log \frac{A_{\text{Na}}}{A_{\text{Cl}}}$$

$$= \frac{RT}{2} \log \frac{A_{\text{Li}}}{A_{\text{Na}}} = \frac{RT}{2} \log \frac{K_{\text{Li}}}{K_{\text{Na}}}.$$

Hence it is evident that the P.D. depends only on the difference in the "true" partition coefficients,[20] or the solution tensions, and that if

[18] Haber, F., *Ann. Physik*, 1908, xxvi, series 4, 927. Haber, F., and Klemensiewicz, Z., *Z. physik. Chem.*, 1909, lxvii, 385.

[19] *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i, pp. 186, 190.

[20] The formula of Nernst has been extended to solutions containing more than one salt by Michaelis, L., and Fujita, A., *Z. physik. Chem.*, 1924, cx, 270, and by Horovitz, K., *Z. physik. Chem.*, 1925, cxv, 424. Thus for a mixture of NaCl and KNO_3 in water the formula would be

$$\text{P.D.} = \frac{RT}{2} \log \frac{A_{\text{Na}} C_{\text{Na}} + A_{\text{K}} C_{\text{K}} + A_{\text{H}} C_{\text{H}}}{A_{\text{Cl}} C_{\text{Cl}} + A_{\text{NO}_3} C_{\text{NO}_3} + A_{\text{OH}} C_{\text{OH}}}.$$

Li has a greater tendency to enter than Na, LiCl will be negative to NaCl.[21]

That these formulæ do not account for the concentration effect may be made clear by an illustration. If we apply LiCl at one spot in the concentration C_1 and at another in the concentration C_2 we have at one place P.D. $= RT \log \left(\frac{C_{1Li}}{C'_{1Li}}\right) K_{Li}$ and at the other P.D. $= RT \log \frac{C_{2Li}}{C'_{2Li}} (K_{Li})$. The total P.D. will be the difference between these or

$$\text{P.D.} = RT \log \left(\frac{C_{1Li}}{C'_{1Li}}\right) - RT \log \left(\frac{C_{2Li}}{C'_{2Li}}\right) = RT \log \left(\frac{C_{1Li}}{C_{2Li}}\right)\left(\frac{C'_{2Li}}{C'_{1Li}}\right).$$

[21] In order to visualize the situation it may be convenient to assign fictitious values which satisfy the requirements. This may be done as follows:

	B		*C*	
In X. "True" or "ideal" concentration	Li = 100	Cl = 25	Na = 36	Cl = 25
In X. Actual concentration (= C')	Li = 50	Cl = 50	Na = 30	Cl = 30
In external solution. Actual concentration (= C)	Li = 1	Cl = 1	Na = 1	Cl = 1

In this case LiCl is applied at B (concentration = 1) and NaCl (concentration = 1) at C: $a_{Li} = 100$, $a_{Na} = 36$, and $a_{Cl} = 25$. At B the P.D. $= RT \log 100/50$ and the positive current tends to flow from the external solution into X since the concentration of Li in X is only 50 and its tendency is to push in until the "true" value of 100 is reached; on the other hand Cl tends to leave X since its concentration is 50 and it tends to move out to attain its "true" value of 25, and in consequence the P.D. $= -RT \log 25/50$. Na at C acts in the same way as Li at B, but the P.D. = RT log 36/30. The total P.D. will be found by subtracting that at C from that at B, or

$$\text{Total P.D.} = RT \log \frac{C_{Li}A_{Li}}{C'_{Li}} - RT \log \frac{C_{Na}A_{Na}}{C'_{Na}} = RT \log \left(\frac{100}{50}\right)\left(\frac{30}{36}\right) = RT \log \frac{5}{3}.$$

This is evidently equal to $RT \log \sqrt{\frac{A_{Li}}{A_{Na}}} = RT \log \sqrt{\frac{100}{36}} = RT \log \frac{10}{6} = RT \log \frac{5}{3}$ as above. In this instance A_{Li} and A_{Na} are for convenience put greater than unity, but in an actual case we should expect them to be very much less than unity.

We should expect $\frac{C_{1Li}}{C'_{1Li}}$ to equal $\frac{C_{2Li}}{C'_{2Li}}$ and $\frac{C_{1Li}}{C_{2Li}}$ to equal $\frac{C'_{1Li}}{C'_{2Li}}$, so that the P.D. would be zero. It is evident that this would be the case since we can write

$$\text{P.D.} = RT \log \sqrt{\frac{A_{Li}}{A_{Cl}}} - RT \log \sqrt{\frac{A_{Li}}{A_{Cl}}} = 0.$$

This gives no concentration effect.

According to Wosnessnensky[22] it is possible to account for the concentration effect by supposing that the partition coefficients of the ions are not constant but vary independently with the concentration. In this case $\frac{C_{1Li}}{C'_{1Li}}$ would not be equal to $\frac{C_{2Li}}{C'_{2Li}}$. If we use the formula of Nernst it is easy to show that the sign of the dilute solution will depend on the relation between A_{Li} and A_{Cl}. If we assume for convenience that A_{Li} is always greater than A_{Cl} and that the latter remains constant while the former varies with concentration it is a simple matter to demonstrate that when A_{Li} is greater in the concentrated than in the dilute solution the latter will be positive (and *vice versa*).

Michaelis[23] states that a concentration effect is possible when a second electrolyte is present.

Since Haber and Klemensiewicz[18] found a concentration effect with H^+ ions in the case of certain kinds of glass[24] they assumed that a small amount of water is present in the glass giving a constant concentration of H^+ and OH^- ions. In that case we should have inside the glass $C'_{1H} = C'_{2H}$ and the equation would become

$$\text{P.D.} = RT \log \frac{C_{1H}}{C_{2H}}$$

which would explain the concentration effect.

To account for the concentration effect of various non-aqueous

[22] Wosnessensky, S., *Z. physik. Chem.*, 1925, cxv, 405.

[23] Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i, 205.

[24] According to Horovitz this is not equally true of all kinds of glass (see Footnote 28).

liquids Beutner[25] assumes that an approximately constant concentration of certain ions results from a chemical reaction. Thus if we suppose X to contain an organic acid HA in very small amounts, the reaction

$$\mathrm{LiCl} + \mathrm{H}A \rightleftharpoons \mathrm{Li}A + \mathrm{HCl}$$

might occur.[26] If X contained equal numbers of Li^+ and Cl^- ions there would be no resulting P.D., but if HCl is less dissociated in X than is LiA the number of Cl^- ions would be less and a P.D. would result, which according to Beutner could be calculated by means of the formula

$$\text{P.D.} = RT \log \frac{C_{1\mathrm{Li}}}{C_{2\mathrm{Li}}}.$$

In order to employ this formula it is necessary to assume that HA is present in such small amounts[27] that practically all of it is converted to LiA even when the cation is present in the external solution in exceedingly low concentrations. This would give an approximately constant concentration of Li^+ in X.

The scheme proposed by Beutner involves a number of assumptions, in part tacit, which cannot be discussed here. Some of these assumptions are of very doubtful validity.[26]

If Beutner's scheme[28] (as presented by Michaelis[29]) should be applied to a series of chlorides, A, B, C (of the same molar concentration) such that A is negative to B, and B is negative to C, it would be said that the cation of A tends to be taken up more than that of B (since A is nega-

[25] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920. *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i.

[26] Michaelis and Perlzweig have raised a serious objection to this assumption (*cf.* Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926–27, x, 575). There are other serious objections to Beutner's scheme.

[27] It is assumed that A comes out into the water to a slight extent only.

[28] In applying the equations for phase boundary potential we do not assume that the cell has reached complete equilibrium with the exterior, since in a living growing cell this is not to be expected, but it is possible to assume that the penetrating substance very quickly reaches approximately the equilibrium concentration at the surface or just inside the surface of X, in which case we should have approximately the value demanded by the equations.

[29] Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i. 191 ff. *Cf.* also Foot-note 26.

tive to *B*) and that of *B* tends to be taken up more than that of *C*. (This is on the assumption made by Beutner that diffusion potentials are negligible.) Hence we must suppose that the cations of *A* and *B* penetrate *X* even if that of *C* is unable to do so. In this way it might be possible to determine what ions[30] enter *X*.

The glass used by Haber and Klemensiewicz acted as a hydrogen electrode only, but Horovitz[31] has found glasses which can take up silver and other cations and act as reversible silver electrodes, etc. This reminds us of the behavior of protoplasm which can act as a reversible electrode for many kinds of ions. The question arises whether the theory[32] formulated by Horovitz for these glasses can be applied to protoplasm. It would require us to assume that the concentration of ions in *X* cannot exceed a certain constant value which is independent of the nature of the ions and that no anions enter except combined with cations in the form of molecules which cannot dissociate in *X* (this does not imply that the substances in question are not wholly dissociated in the external solution, since we may assume that ions combine at the surface of *X* to form molecules and so pass through *X*). We should have to assume that the cell gives out as many cations as it takes up, but it is of course possible that it can produce enough H^+ ions for this purpose.

The conclusions already drawn regarding the series *A*, *B*, *C* would remain unchanged on the basis of the scheme proposed by Horovitz, providing diffusion potentials are neglected; this however is not permissible, according to Horovitz, so that we cannot tell which cation tends to be taken up to a greater degree, but we can say in regard to the series *A*, *B*, *C* that the cation of *A* tends to be taken up more than that of *B* or else has a greater mobility in *X* (or that both statements are true). On either basis we should conclude that the cation of *A* is able to enter *X*.

[30] Whenever the entrance or taking up of ions is mentioned it is of course understood that effects may be produced by the exit of these ions.

[31] Horovitz, K., *Sitzungsber. Akad. Wissensch. Wien, Math.-naturw. Kl., 2a Abt.*, 1925, cxxxiv, 335; *Z. Physik*, 1923, xv, 369. Horovitz, K., and Zimmermann, J., *Sitzungsber. Akad. Wissensch. Wien, Math.-naturw. Kl., 2a Abt.*, 1925, cxxxiv, 355.

[32] This is still unpublished. I am indebted to Dr. Horovitz for the privilege of seeing his manuscript in advance and for discussion of the theories here considered.

Let us now consider the Donnan potential.[33] As already stated[28] it may be doubted whether any part of an actively growing cell can come into a condition of real equilibrium with its surroundings and it could not very well be in equilibrium with two different solutions applied at different places. The question arises whether an approximate local Donnan equilibrium might be set up at two different points in contact with different concentrations of the same salt, so that we could calculate the P.D. by means of the usual formula

$$\text{P.D.} = RT \log \frac{C_1}{C_2},$$

where C_1 is the concentration of a diffusible cation in the external solution and C_2 its concentration inside the membrane.

If this were the case we might expect a concentration effect which would fall off with increase of concentration (as is the case with protoplasm[34]). But, as has been pointed out by Michaelis,[35] we should *not expect this to be as large as that observed in the cell. If such* an effect exists it seems very doubtful whether it can be calculated in this way since there are disturbing factors, such as movement of water due to osmotic pressure, etc.

On the other hand, it is difficult to see how a chemical effect could arise since at equilibrium all the diffusible cations would be expected to behave alike. They might, of course, differ in speed of penetration or in activity, but it is a question to what extent a temporary chemical effect could arise in this way. If it exists we should still conclude that if A is negative to B (in the series mentioned above) it means that more cations of A are taken up.

[33] This is variously classified by different authors but may for convenience be placed in a separate category. *Cf.* Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i. Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926–27, x, 575.

[34] The (unpublished) formula proposed by Horovitz, as well as that employed by Beutner, would lead us to expect an increase in concentration effect (*i.e.* an increased increment in potential difference for a fivefold dilution) as the concentration increases from zero, but after a certain point is reached no further increase in the concentration effect would be expected. It is found both with protoplasm and with the organic liquids immiscible with water studied by Beutner that the concentration effect falls off as the concentration increases above a certain point.

[35] Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, i.

We cannot decide at present to what extent bioelectric effects are to be attributed to diffusion potentials, to phase boundary potentials, or to Donnan potentials. It seems probable that in most cases two or more of these act simultaneously. We can, however, arrive at certain conclusions regarding the penetration of ions, provided we adhere to any of the schemes discussed above. Regarding the series of chlorides A, B, C previously referred to, we can say that the cations of A and B must be able to penetrate. For on the basis of any of the hypotheses outlined above we can say that even if the cations of C cannot enter, those of B must go in in order that B may be negative to C. Conversely, if we have a series of K salts D, E, F (of the same concentration), with D positive to E and E positive to F, we can say that the anions of D and E penetrate even if those of F do not.

We can also say that where there is a concentration effect not due solely to the cell wall ions must be able to enter the protoplasm.

Let us now consider the possibility of measuring the absolute values of certain potential differences. It seems probable that in brief experiments the applied salt solution does not penetrate through X into the deeper layers, and that in consequence any changes observed are due to changes in X. Let us suppose that we lead off from two places, B and C, and measure the potential difference of C against B[12]. Since the potential difference of B is opposite to that of C in the circuit, we may write

$$\text{Observed P.D. of } C = (a_C + Z_C) - (a_B + Z_B),$$

where a_C is the absolute value of the potential difference at the surface of X (a, Fig. 2) at the point in contact with C, and Z_C is the sum of the remaining values in X and in the deeper layers (the values of a_B and Z_B have corresponding significance).

If at the point in contact with B anions and cations tend to enter X to about the same degree, the value of a_B may be negligibly small and we shall have

$$\text{Observed P.D. of } C = a_C + Z_C - Z_B.$$

If the values of Z are the same at all points in the cell (assuming that the applied salt solution has not yet penetrated through X) this reduces to

$$\text{Observed P.D. of } C = a_C.$$

In this case we might be able to approximate the absolute value of a_C.

If we obtain the absolute value of the potential difference across the protoplasm at C, and if, as before, we write P.D. $= a + Z$, it is evident that if the value at a is negligibly small we may be able to approximate the value of Z. If it should happen (a possibility suggested above) that neither anions nor cations enter Y, the potential difference at both c and d might be zero and we should be able to approximate the value of $b_C + g + h$.

It seems evident from what has been said that bioelectrical investigations may throw some light upon the structure and properties of protoplasm. An especial advantage of this method of study is that it enables us to detect and record changes which last only a fraction of a second. It may thus uncover important activities of the protoplasm which would otherwise escape observation on account of the crudity of our methods of observation. This will be fully discussed in later reports.

SUMMARY.

It is pointed out that there are great advantages in using single cells instead of tissues in the study of bioelectrical phenomena.

Certain bioelectrical phenomena are discussed in relation to the structure of protoplasm.

Under certain circumstances measurements of potential differences may enable us to determine what ions enter the protoplasm.

Under suitable conditions we are able to ascertain the potential differences across the protoplasm at single points, instead of being obliged merely to measure the differences between two points.

ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume is appearing simultaneously with Volumes IX and X. Number 1 of this volume will contain a biography of Dr. Loeb. It is to appear after Number 6, and the page numbers will be roman numerals. The publication of this volume began September 18, 1925.

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COMBINATION OF GELATIN WITH SOME ORGANIC BASES.

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(Accepted for publication, June 5, 1927.)

It is known that the alkaloids are very strong poisons. Their poisoning power can be compared only with that of the glucosides, the hormones, the saponins, HCN, and some bacterial toxins. The alkaloids are sufficiently studied physiologically, and in relation to their chemical structure, but what chemical substances enter in combination with them in the body and in the cell is wholly obscure. Traube explains all the influence of alkaloids through changes in superficial tension. Many investigators have adopted the theory of Overton—that alkaloids, which are all soluble in lipoids, influence the cell through this solution. This hypothesis is supported by the fact that the nervous tissue, so greatly affected by the alkaloids, contains at the same time the greatest amount of lipoids. Other investigators, such as Brunton, Rossbach, and lately Frey and Gürber, think that the alkaloids can combine with the proteins. The latter indicate that the alkaloids can precipitate the proteins. But the conditions of this combination are very incompletely known. Woronzoff says that the liver retains the alkaloids only when Ringer-Locke solution, that contains the alkaloid, is alkalized. When it becomes acid, the liver releases the alkaloid previously retained. He thinks that the acid solution extracts proteins which combine with the alkaloids; but then the question arises,—can the proteins be restored to their former place by making the solution alkaline?

In his book, "Proteins and the theory of colloidal behavior," Loeb gives a very simple method for the investigation of the influence of the pH of the solution on the combination of proteins with anions or cations. He used powdered gelatin that acquired different pH values by putting it for 1 hour in acid solutions of different concentration, then filtering off, washing, and putting in an equal quantity of the

solution of the salt under study. He used $AgNO_3$, $K_4Fe(CN)_6$, and other cations and anions, *e.g.* neutral red, acid and basic fuchsin. In all experiments he came invariably to the same result—the anions are combined only on the acid side of the isoelectric point of the gelatin, the cations only on the basic side.

It seemed of some interest to us to investigate by the same method the alkaloids which form a separate group through their common basic character.

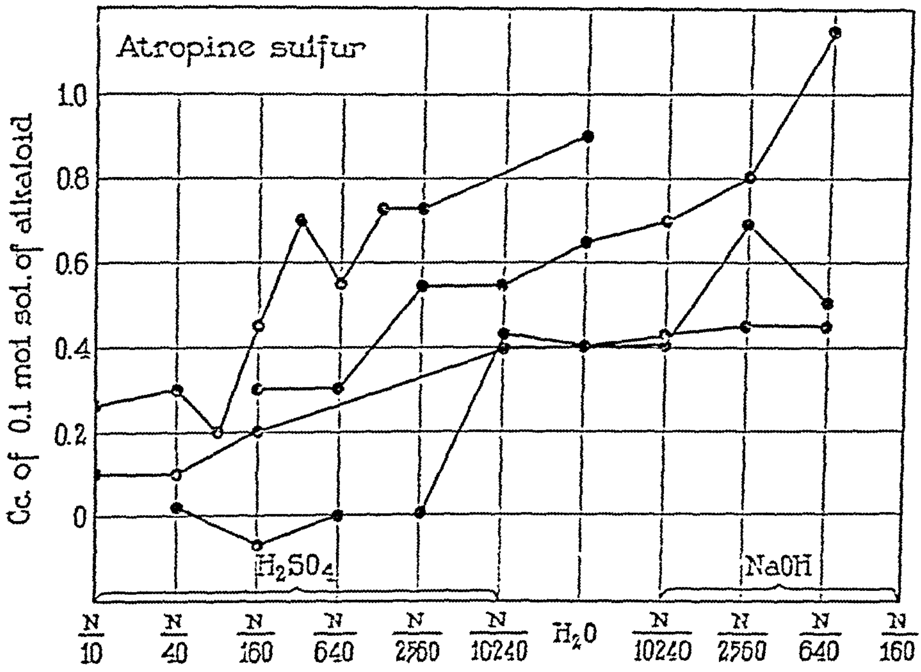

FIG. 1. Curves for atropine. Abscissæ, normality of acid or base used to bring the gelatin to different pH. Ordinates, cc. of M/10 alkaloid fixed by the gelatin determined by difference of alkaloid added and found in the filtrates and wash-waters.

Loeb's method, so very satisfactory in one way, in another way cannot be used for the alkaloids. He determined the combination of anions or cations with the gelatin by its coloring. But the alkaloids are colorless. This forced us to determine them by titration of the filtrates and wash-waters from the known solution of alkaloid, which remained for 1 hour in contact with the gelatin in question. We assembled them all, separately for each sample of gelatin, and titrated

the alkaloid with NaOH and phenolphthalein in presence of any organic solvent, chloroform, or alcohol for atropine, benzene (benzol) for quinine and strychnine, by continued energetic shaking. The added NaOH releases the alkaloid from its salt, the alkaloid dissolves in the organic solvent, and the mixture remains colorless. A new addition of NaOH forces a new quantity of alkaloid to pass in the solvent, and this continues till the last traces of alkaloid pass into the solvent. The following addition of alkali makes the mixture red. This method gives very good results with solutions that contain only alkaloids, but we possessed a mixture of alkaloids with acid or base, that passed into the solution from previous treating of the gelatin. We determined

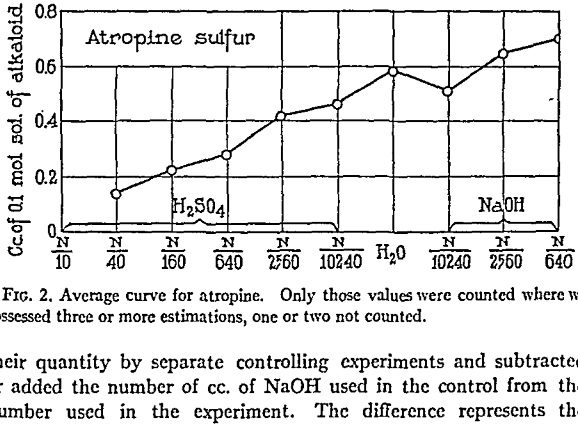

FIG. 2. Average curve for atropine. Only those values were counted where we possessed three or more estimations, one or two not counted.

their quantity by separate controlling experiments and subtracted or added the number of cc. of NaOH used in the control from the number used in the experiment. The difference represents the quantity of alkaloids in the solution. The difference between this quantity and that added represents the alkaloid quantity combined with the gelatin. The accompanying charts indicate very clearly that gelatin combines with the alkaloids very much on the basic side of its isoelectric point. On the acid side the combination is very little or none. The quinine curve gave apparently a very strange result: in all our experiments on the acid side, we found more alkaloid than was added. We think it is because the titration method

does not determine the alkaloid itself, but the acid in combination with it that the quantity of this acid is increased on the acid side. Quinine can form two sorts of salt—one with one or two parts of acid; on the acid side of the isoelectric point in this case we possess a mixture of two bases, the monochlorate of quinine and the gelatin—and the other a single acid, the H_2SO_4 in combination with the gelatin. The basic character of the gelatin is more feeble than that of the quinine salt

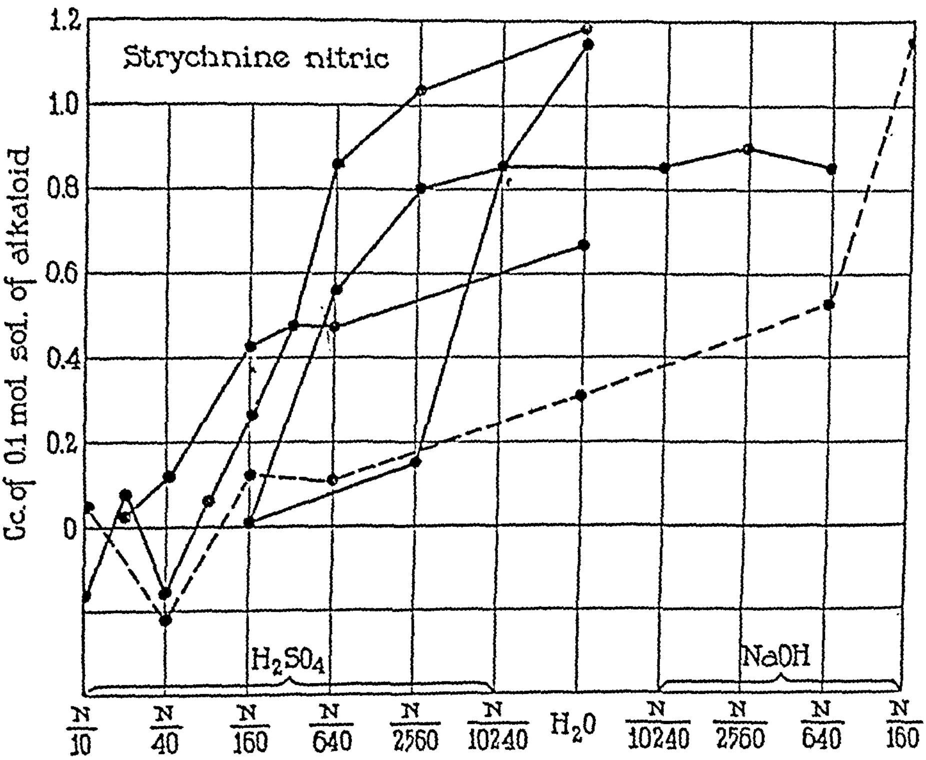

FIG. 3. Curves for strychnine. Determined by the same method as for atropine.

and the latter subtracts a part of the acid from the former. This process is expressed through the lower part (under 0) of the quinine curve.

But the question arises that perhaps this disappearance of alkaloids from the solution is to be explained by their destruction and not by their fixation. This is very unlikely because strychnine and quinine are both very stable substances, but we think that this supposition

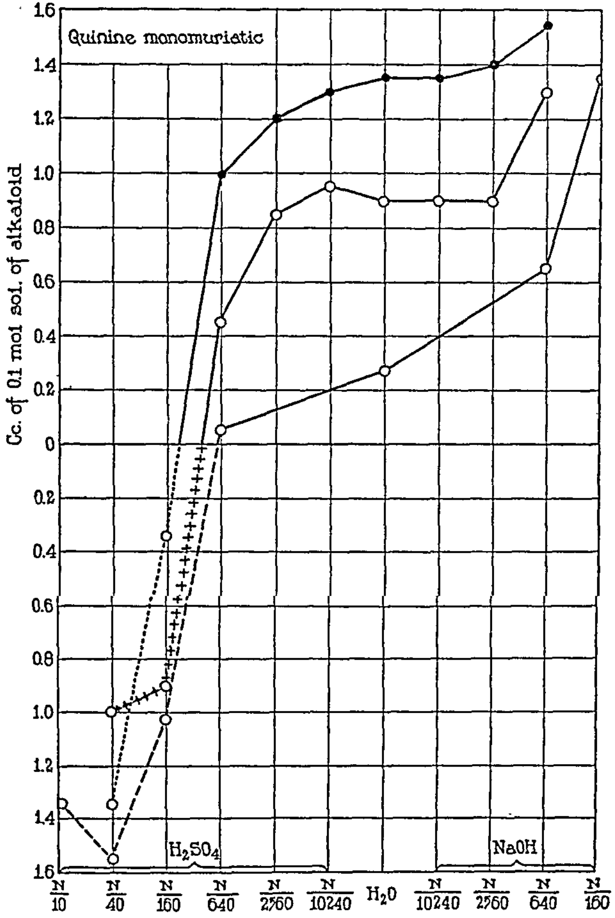

Fig. 4. Curves for quinine. Determined by the same method as for atropine. The curve below 0 indicates the number of cc. of acid extracted by quinine from the gelatin.

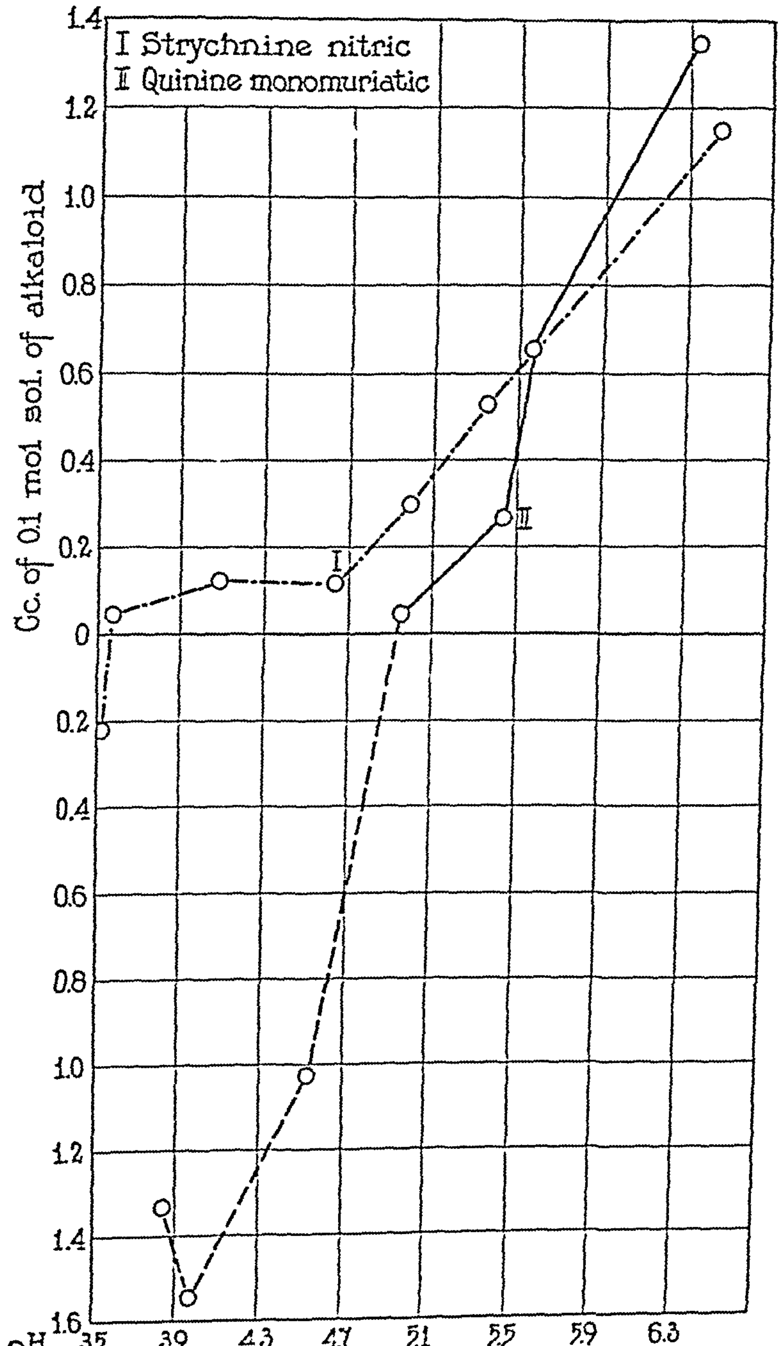

FIG. 5. Curves for strychnine and quinine (each average of two estimations). Abscissæ, pH determined electrometrically, cc. of alkaloid fixed determined by detitration.

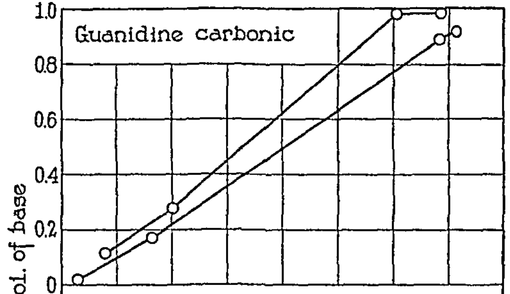

FIG. 6.

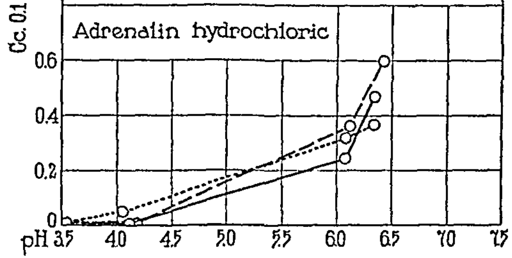

FIG. 7.

FIG. 6. Curves for guanidine (each curve is an average of two estimations). Guanidine determined in the filtrates and wash-waters by the Kjeldahl method; the number of N found divided by 3 (3 atoms of N in each molecule of guanidine) and subtracted from the number of cc. added. Correction given for the N of gelatin.

FIG. 7. Curves for adrenalin (each curve is an average of two estimations). Adrenalin determined in the filtrates and wash-waters by the method of Folin. The studied solution used as standard. No correction for N gelatin (it does not give the Folin reaction). Curves for the difference of adrenalin added and adrenalin found.

can be verified very easily. We tasted the gelatin, because quinine and strychnine are both very bitter. The acid gelatin was not bitter at all even if alkalized, and the basic was bitter. The bitterness grew with the basicity. The gelatin used in experiments with atropine was investigated for its power to dilatate the pupil; the acid did not act at all and the basic caused a very distinct and prolongated dilatation of the pupil of young dogs.

But besides alkaloids there exist other bases, the so called animal bases, and many of them are of a very marked and great significance for the organism. We thought that they should give the same results, and verified this supposition by the same experiment. We chose guanidine, the rôle of which in tetany is so much disputed, and adrenalin, the significance of which in the organism is unquestioned. We determined the guanidine in the filtrates by the method of Kjeldahl—a correction being given for the N from the gelatin itself. As shown by the curve, the results are the same as in preceding experiments. The adrenalin was determined by the method of Folin, the adrenalin solution used being employed as standard, with the same results. Correction for the gelatin was not necessary, since the filtrate from the gelatin used did not give the Folin reaction. But adrenalin is very rapidly destroyed in basic solution, and it was necessary to ascertain that its disappearance from the solution was not caused by destruction, but by fixation. We used a physiological test. The gelatin in question was placed on the conjunctiva of the eye—the basic gave a rapid constriction of the vessels, the acid, none.

We think that the same results will be given by histamine, spermine, and other animal bases, and that the experiments here described give us some right to affirm that alkaloids and the so called animal bases can combine with the proteins (with gelatin we have had an experiment with a mixture of brain proteins and strychnine with the same result) only on the basic side of their isoelectric point. On the acid side the combination is very slight if any. The same must be true in the living cell. The experiments of Bornstein and Rüter and of Labes, explained by the authors in some other manner, reproduced our results so exactly that this cannot be a mere coincidence. In the experiments of Labes, tadpoles swim in the acid solution of atropine or cocaine without any signs of poisoning; in the basic solution they

perished very rapidly. Neuschlosz in his experiments with veratrine found that a gelatin plate, that retained veratrine from previous treating, releases more alkaloid when placed in an acid solution, than in a basic solution. We think the characteristic curve of muscle contraction after veratrine application can be explained in this manner. The muscle tissue under the influence of veratrine gives very characteristic contracture for a certain time, after which its contraction becomes normal. When the muscle is allowed to rest, its contraction returns to its former character but after some further contractions becomes again normal. *We think this can be explained by the fact that contraction* of muscle is followed by appearance of lactic acid, which lowers the pH of the muscle below the isoelectric points of its proteins (their isoelectric point is very high according to Weber—6.3 for myogen and 5.15 for myosin), and below this value the protein cannot fix the alkaloid. When the muscle rests, the acid disappears, the pH is raised above the isoelectric point, and the muscle fixes the alkaloid, and the latter influences its contraction.

We think that the experiments of Neuschlosz and Riesser with the heart glucosides can be explained in the same manner with the difference only that these substances in contradistinction to the alkaloids are acid and are combined on the acid side of the isoelectric point. *The chemical structure of these glucosides is very insufficiently* studied, it is better known only for strophanthin, and it contains in its molecule a lactone of an acid. We think it of some interest to study this question more closely. In a recent number of the *J. Biol. Chem.* a very interesting communication by Chapman, Greenberg, and Schmidt appeared, who studied the fixation of acid dyes by gelatin, edestin, casein, and deaminised gelatin. They came to the same result as Loeb—that these dyes can be fixed only on the acid side of the isoelectric points of these proteins, and that this fixation possesses a stoichiometric character, and, what is new, stands in a very close relation to the quantity of the diamino-N of these proteins. By deaminising the proteins their combining power for acid dyes can be greatly lowered. We think this is a very strong proof against the adsorption theory.

The same method can give interesting results in the investigation of the toxins, and this we expect to undertake.

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THE EFFECT OF ACETATE BUFFER MIXTURES, ACETIC ACID, AND SODIUM ACETATE, ON THE PROTOPLASM, AS INFLUENCING THE RATE OF PENETRATION OF CRESYL BLUE INTO THE VACUOLE OF NITELLA.

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(Accepted for publication, June 5, 1927.)

An adequate theory of the penetration[1,2,3] of cresyl blue into the vacuole of living cells of the fresh water plant *Nitella* may be stated as follows. The dye, in form of free base, which predominates at a high pH value, diffuses in and out of the vacuole very freely, while the dye in form of salt, which predominates at a low pH value, diffuses so slowly that its rate of penetration and that of the exit may be neglected in experiments of the present type. Upon the entrance of free base into the vacuole it is partly converted at once into the salt (the extent depending on the condition of the sap, *i.e.* the pH value). The concentration of free base inside the vacuole is always proportional to that of the free base in the external solution.

We may picture the entrance of dye into the vacuole as a reversible process[2] depending primarily on the diffusion of free base through a very thin layer of protoplasm which lies between the cell wall and an inner central vacuole filled with aqueous sap (at about pH 5.5 and containing about 0.1 M halides). The consideration of cell wall may be neglected since the substances in this case do not seem to affect it enough to change the rate of penetration of dye into the vacuole.

[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561; *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 251.

[2] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 75.

[3] The following investigators have stated that basic dyes in form of free base penetrated living cells: Overton, E., *Jahrb. wissensch. Bot.*, 1900, xxxiv, 669; Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507; Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1.

The protoplasm may therefore be treated here as if it were in direct contact with the external aqueous solution. The protoplasm proper[2] may be assumed to consist of (1) an outer non-aqueous layer[4] in contact with the external solution, (2) a middle aqueous layer, (3) an inner non-aqueous layer surrounding the central vacuole, and (4) their respective surface boundaries.

Thus the penetration of basic or acid dye into the vacuole may depend (1) on the ease with which each non-aqueous phase takes up the dye from one aqueous phase and gives it up to the other aqueous phase, (2) on the condition of the surface boundaries at the outer and inner non-aqueous layers, and (3) on the ratio of free base or free acid to salt in the aqueous phases in case the solubility of free base or free acid in non-aqueous layers differs from that of the salts. Thus there are to be considered two partition coefficients of a dye between non-aqueous (behaving like a lipoid) and aqueous phases at each protoplasmic boundary (external and vacuolar). It is uncertain as to whether the rate of penetration of dye is controlled by both boundaries or by one only. This theory serves to explain why many basic dyes enter the vacuole more readily in form of free base than salt, while some basic or acid dyes even in form of free base or free acid, as well as some other substances in dissociated or undissociated forms do not readily enter the vacuole of living cells. A detailed account will be subsequently published.

An alteration in any of these parts may change the rate of penetration if the rate is controlled by that particular part of the cell.

It may be possible therefore to locate the factors controlling the rate by changing the conditions in the various ways outside and inside the cell.

It was shown[1,2,5] previously that by altering the external solution (*viz.*, the pH value), it was possible to change the rate of penetration of dye.

An alteration in the protoplasm by various substances was previously found to decrease the rate of penetration of cresyl blue.

Experiments have been made[6,7] showing that decrease in the rate of penetration of dye occurred when the pH value of the sap was raised by the entrance of ammonia. But such experiments do not necessarily prove that the decrease in the rate of penetration of dye is caused by the change in the pH value of the sap, since they do not show that the rate is not controlled by a change in the protoplasm. In fact it was found[7] that a decrease in the rate of penetration of dye took place even before a measurable rise in the pH value of the sap occurred. This decrease

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[4] Overton claimed that dyes soluble in lipoid penetrated the living cells (*cf.* Foot-note 3).

[5] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 427; 1925–27, viii, 147.

[6] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

[7] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.

in the rate may therefore be due to the increase in the pH value of the protoplasm, or to some other effect of ammonia or ammonium salt on the protoplasm (either at the surface or in the interior). Since there is no way of settling this experimentally, it is not possible to draw any final conclusion as to the cause of the decrease in the penetration of dye. Thus these experiments do not seem to prove or disprove the theory presented.

The supposition that the factor controlling the rate of penetration of dye might be located in the protoplasm under certain conditions is supported by the following experimental results. When cells are exposed to either (1) sodium chloride[8] or (2) phosphate buffer solution,[9] and are then placed in brilliant cresyl blue solution, the rate of penetration of dye into the vacuole is decreased, without any change in the sap. This decrease is found to be caused by the action on the protoplasm (1) of sodium present in sodium chloride, and (2) of monovalent base cations and phosphoric acid present in the phosphate buffer mixture.

If cells are exposed to an acetate buffer mixture,[9] in which the pH value of the sap is lowered, a decrease in the rate of penetration of dye takes place. If this decrease were actually caused by the lowering of the pH value of the sap, then the present theory of penetration of dye would have to be revised. But if this decrease is brought about by the action of acetate buffer mixture on the protoplasm, rather than by the changes in the pH value of the sap, such a result would not necessarily discredit the theory. In order to determine this point it is necessary to compare the behavior of the respective constituents of the acetate buffer mixture, namely acetic acid and sodium acetate, and to show if hydrogen ions, at the pH value used, play any part. The data and discussion of these experiments are reported in the present paper.

II.

Method.

The cells employed were collected near New York, but not in the same place as those used in previous experiments. Owing to the mild winter and sheltered

[8] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 425; *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 54.

[9] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

locality, the cells were still in the same condition as they were in the early fall though these experiments were made at the beginning of winter. For convenience we shall call these *Nitella* New York II, as contrasted with the New York *Nitella* employed for the experiments described in some of the previous publications. So long as a series of comparative experiments was made within a few days with the same lot of cells it was possible to obtain consistent results.

Since the methods of choosing the best type of cells, of detecting the condition of the cells during the experiment, and of colorimetric determination of the dye in the sap have been described in detail in the writer's previous publications,[2,5,7] they will be omitted here. Great care was taken to keep the cells from injury during the experiments. The experiments were carried out in an incubator at 25 ± 0.5°C., and the dye used was prewar Grübler's brilliant cresyl blue. In all cases the buffer mixtures used were made up from Clark's[10] table; M/15 phosphate buffer mixture was diluted ten times, and acetate buffer mixture was diluted thirty times.

The dye solution at pH 6.79 was made up with M/15 phosphate buffer mixture at pH 6.64 diluted ten times, and at pH 7.85 was made up with either M/15 phosphate buffer mixture at pH 7.7 or borate buffer mixture (M/5 boric acid and M/20 borax) at pH 7.36, diluted ten times.

The pH values of the solutions of buffer mixtures were determined by means of the hydrogen electrode; the pH values of acetic acid, sodium acetate, and hydrochloric acid were determined colorimetrically. The pH values of the sap were determined by use of methyl red.

In all cases the cells were wiped, dipped in distilled water for 5 seconds, and again wiped before they were placed in the dye solution. The cells were always wiped before they were placed in any solution.

Each experiment represents an average of over 100 readings on individual cells. The probable error is less than ± 6 per cent of the mean.

III.

When the Dye Solution was Made up with Phosphate Buffer at pH 6.79.

It was necessary first of all to repeat some of the experiments previously made,[9] since the cells employed here were not of the same lot as those used before.

When cells were placed (without stirring) for 10 minutes in phosphate or acetate buffer solution at pH 5.5 and then placed in brilliant cresyl blue solution made up with phosphate buffer mixture at pH 6.79, there was 30 per cent decrease in the rate of dye penetration with acetate and 23 per cent decrease with phosphate (Table I).

[10] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, pp. 81–83.

These results confirm those previously published,[9] except that the percentage decrease is lower; in former experiments the acetate gave 47 per cent decrease and the phosphate 44 per cent decrease. This difference in the percentage decrease with these two groups of cells may be due to difference in the locality as well as in the season of the year.

When cells were exposed to acetate buffer mixture at pH 5.5 for 10 minutes, the pH value of the sap decreased from pH 5.5 (normal) to pH 5.0, while phosphate buffer mixture brought about no change in the pH value of the sap.

TABLE I.

Comparison of the amount of dye found in the vacuole of living cells of *Nitella* when cells were first exposed for 10 minutes either to an acetate or phosphate buffer solution at pH 5.5 and were then placed for 1 minute in 34.5×10^{-5} M brilliant cresyl blue at pH 6.79 made up with phosphate buffer mixture. The solutions were not stirred. The buffer mixtures are described in Section II in the text.

External solution to which cells were exposed	Tap water or control	Phosphate buffer at pH 5.5	Acetate buffer at pH 5.5
Amount of dye in sap, $M \times 10^5$	19.7	15.2	13.8
Percentage decrease or increase	Standard	23 per cent decrease	30 per cent decrease
pH value of the sap before the cells are placed in the dye solution	5.5	5.5	5.0

Since the inhibiting effect is brought about irrespective of the changes in the pH value of the sap, we may conclude as before[9] that the factor controlling the rate of dye penetration may not lie in the vacuole, in this case, but that it lies in the protoplasm (either at the surface or the interior).

IV.

When the Dye Solution is Made up with Borate Buffer Mixture at pH 7.85.

When uninjured cells of *Nitella* were placed in various solutions and after 10 minutes the pH value of the sap was determined, the results

TABLE II.

Comparison of the amount of dye found in the vacuole of living *Nitella* when cells were first exposed for 10 minutes to one of the various solutions given below and were then placed for $\frac{1}{2}$ minute in 13.8×10^{-5} M brilliant cresyl blue solution at pH 7.85 made up with diluted borate or phosphate buffer mixture. The pH values of the sap after cells were exposed for 10 minutes to these various solutions are given below.

Buffer mixtures are described in Section II in the text.

			Tap water or control	Phosphate buffer mixture	Acetate buffer mixture	Acetate buffer mixture	Acetic acid	Acetic acid	M/150 sodium acetate	Hydrochloric acid	Hydrochloric acid
				pH 5.5	*pH 5.5*	*pH 4.8*	*pH 4.8*	*pH 4*	*pH 7.1*	*pH 4.8*	*pH 4.2*
Dye solution made up with borate buffer mixture	Dye solution stirred	Amount of dye in sap, $M \times 10^5$	41.4	19.3	15.5	10.7	37.6	11.4	18.5	37.7	30.0
		Percentage decrease or increase	Standard	53 per cent decrease	63 per cent decrease	74 per cent decrease	9 per cent decrease	72 per cent decrease	55 per cent decrease	9 per cent decrease	28 per cent decrease
Dye solution made up with phosphate buffer mixture	Dye solution stirred	Amount of dye in sap, $M \times 10^5$	40.7	38.7	19.0	15.9		20.0	36.6		43.7
		Percentage decrease or increase	Standard	5 per cent decrease	53 per cent decrease	61 per cent decrease		51 per cent decrease	10 per cent decrease		8 per cent increase
	Dye solution not stirred	Amount of dye in sap, $M \times 10^5$	22.4	22.8	12.1						
		Percentage decrease or increase	Standard	2 per cent increase	46 per cent decrease						
pH values of the sap...............			5.5	5.5	5.0	4.8	5.2	4.9	5.5	5.5	5.5

given in Table II were obtained. In phosphate buffer mixture, and M/150 sodium acetate, the pH value of the sap remained unchanged, while in acetate buffer mixture and in acetic acid the pH value of the sap decreased. In either acetate buffer mixture or acetic acid the lower the external pH value, the greater the decrease in the pH value of the sap but the extent of decrease was greater in acetate buffer mixture at pH 4.8 than in acetic acid at the same pH value. This difference may be due to the difference in the supply of acetic acid. With acetate buffer mixture more acid is formed in the external solution during the experiment, replacing the acid which has diffused into the cell, while with acetic acid this does not take place so that in a given period more acetic acid enters the vacuole from the former than from the latter.

When the cells thus exposed to various solutions were placed in the solution of brilliant cresyl blue made up in borate buffer mixture at pH 7.85, the following results shown in Table II were obtained.

With cells previously exposed to sodium acetate, the rate of penetration of dye was decreased considerably without any change in the pH value of the sap. This decrease seems to be a result of the action of sodium ions on the protoplasm since acetic acid at this pH value has no inhibiting effect.

When cells previously exposed to hydrochloric acid at pH 4.8, were placed in the dye solution, the rate of penetration did not decrease appreciably, as shown in Table II, but at a lower pH value, 4.2, about 30 per cent decrease was brought about. Since previous experiments indicated that the inhibiting effect of various other chlorides was not due to the chloride ions, this effect of hydrochloric acid may be due to the hydrogen ions and not to the chloride ions.

With cells previously exposed to acetic acid the rate of penetration of dye was found to decrease with the lowering of the pH value of the external solution and with the lowering of the pH value of the sap as shown in Table II. For the reason already discussed, the inhibiting effect of acetic acid at pH 4.8 is not due to the hydrogen ions in the external solution but at pH 4 it is partly due to the hydrogen ions and partly to the acetic acid in the external solution. The latter may have a specific effect on the protoplasm or may enter as undissociated molecules and dissociate in the protoplasm thereby

lowering the pH value of the protoplasm. Though the extent of decrease in the rate of penetration of dye corresponds with the lowering of the pH value of the sap, it does not necessarily signify that this decrease is brought about by the lowering of the pH value of the sap. In view of the fact that hydrogen ions and base cations may have an inhibiting effect without changing the pH value of the sap, we may suppose that in the case of acetic acid the decrease in the rate of penetration of dye is brought about by the inhibiting effect of acetic acid on the protoplasm.

With cells previously exposed to an acetate buffer mixture it is found (Table II) that the rate of penetration of dye decreases with the lowering of the pH value of the external solution and that of the sap. In this case the effect of hydrogen ions in the external solution cannot be the chief cause, because at these external pH values there is practically no effect of the hydrogen ions on the protoplasm. Since the acetate buffer mixture is made up of sodium acetate and acetic acid, both of which are found to have an inhibiting effect, the decrease in the rate of penetration must therefore be due to the effect of sodium acetate and acetic acid on the protoplasm.

The fact that at the external pH value of 4.8 the acetic acid has practically no inhibiting effect while the acetate buffer mixture has a considerable inhibiting effect (about 70 per cent) may be explained as due to two factors: (1) the presence of sodium in the acetate buffer mixture which is found to have an inhibiting effect on the protoplasm, and (2) the greater supply of acetic acid in the acetate buffer mixture.

V.

When the Dye Solution is Made Up with Phosphate Buffer Mixture at pH 7.85.

In the writer's previous publication[11] it was shown that when cells were exposed to phosphate buffer mixture at pH 5.5 or to phosphoric acid or to hydrochloric acid at pH 4.2 in which the pH value of the sap remained unchanged, and were then placed in the dye solution made up with phosphate buffer mixture at pH 7.85, the inhibiting effect of these solutions on the protoplasm was entirely masked. It

[11] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926 27, xxiv, 245.

is of interest to see if such a counteraction of the inhibiting effect of acetate buffer mixtures and that of acetic acid in which the pH value of the sap is decreased will likewise take place if cells previously exposed to these solutions are placed in such a dye solution.

Living cells were therefore exposed for 10 minutes to various solutions and were then placed in brilliant cresyl blue solution at pH 7.85 made up with phosphate buffer mixture (Table II). The inhibiting effect described in Section IV was almost completely removed in the case of the phosphate buffer mixture at pH 5.5 and sodium acetate, but only slightly diminished in the case of the acetate buffer mixture and acetic acid, as shown in Table II.

That the inhibiting effect on the protoplasm is only very slightly removed in the case of acetate buffer mixtures and acetic acid may be due to the fact that the acetic acid in the sap serves as a reservoir which enables the inhibiting effect to persist for a time even when the cells are placed in the dye solution made up with phosphate buffer mixture at pH 7.85, while there is no inhibiting substance in store to act as a reservoir with phosphate buffer mixture and with sodium acetate, since these substances do not collect in the vacuole.

The decrease in the rate of penetration of dye in the case of the cells exposed to acetate buffer mixture is not due to the lowering of the pH value of the external solution immediately surrounding the cell, resulting from a diffusion of acetic acid from the cell after the cells are transferred from the acetate buffer mixture into the dye solution, since the rate of penetration of dye is decreased to about the same extent whether the external dye solution is stirred or not (Table II).

VI.

CONCLUSION.

The experiments show that the inhibiting effect of sodium acetate is due to the action of sodium and acetic acid on the protoplasm. Just as in the case of phosphate buffer mixture, this inhibiting effect may be removed when the dye solution is made up with a salt solution containing a certain concentration of base cations.

The inhibiting effect of acetic acid at pH 4.8 is due either to the specific effect of acetic acid on the protoplasm or due to the entrance of

undissociated molecules of acetic acid and their subsequent dissociation in the protoplasm thereby lowering the pH value of the protoplasm. With the lowering of the pH value the concentration of the dye in form of free base decreases in the protoplasm. If, therefore, we assume that the rate of penetration of dye in this case is controlled by the diffusion of dye in form of free base from protoplasm into the vacuole and that the inhibiting effect of acetic acid on the protoplasm exceeds the accelerating effect on the sap in the vacuole, we would expect a decrease in the rate of penetration to occur. Since both a non-aqueous substance and the vacuole take up the dye more readily in form of free base than in form of salt, we may assume that the vacuole is surrounded by a non-aqueous layer ("inner layer of protoplasm" in Section I). The rate of penetration of dye therefore may depend on the amount of free base taken up by this non-aqueous layer from the aqueous layer of protoplasm ("middle layer" in Section I) and given up to the sap in the vacuole, in a given time. If any part of the protoplasm is affected in such a way as to change the absorption and giving up of free base by this non-aqueous layer, the rate of penetration of free base into the vacuole will be accordingly altered.

For reasons already stated in Section III, the inhibiting effect of acetic acid at pH 4 may be considered to be due partly to the hydrogen ions in the external solution.

The inhibiting effect of acetic acid and of the acetate buffer mixture is not completely counteracted in presence of the dye made up with a diluted phosphate buffer mixture at pH 7.85. This may be due to the presence of acetic acid in the vacuole, which acts as a reservoir so that the inhibiting effect on the protoplasm persists until this is used up.

We may conclude that in all these cases the inhibiting effect is brought about by the action of these substances on the protoplasm. Under these conditions therefore the factor controlling the rate of penetration of dye into the vacuole is located in the protoplasm and not in the vacuole.

These experiments do not contradict the theory of penetration of basic dye given in this paper (Section I), although in some cases the rate of penetration of dye appears to decrease with the lowering of the pH value of the sap.

SUMMARY.

When living cells of *Nitella* are exposed to a solution of sodium acetate and are then placed in a solution of brilliant cresyl blue made up with a borate buffer mixture at pH 7.85, a decrease in the rate of penetration of dye is found, without any change in the pH value of the sap. It is assumed that this inhibiting effect is caused by the action of sodium on the protoplasm.

This effect is not manifest if the dye solution is made up with phosphate buffer mixture at pH 7.85. It is assumed that this is due to the presence of a greater concentration of base cations in the phosphate buffer mixture.

In the case of cells previously exposed to solutions of acetic acid the rate of penetration of dye decreases with the lowering of the pH value of the sap. This inhibiting effect is assumed to be due chiefly to the action of acetic acid on the protoplasm, provided the pH value of the external acetic acid is not so low as to involve an inhibiting effect on the protoplasm by hydrogen ions as well. It is assumed that the acetic acid either has a specific effect on the protoplasm or enters as undissociated molecules and by subsequent dissociation lowers the pH value of the protoplasm.

With acetate buffer mixture the inhibiting effect is due to the action of sodium and acetic acid on the protoplasm.

The inhibiting effect of acetic acid and acetate buffer mixture is manifested whether the dye solution is made up with borate or phosphate buffer mixture at pH 7.85. It is assumed that acetic acid in the vacuole serves as a reservoir so that during the experiment the inhibiting effect still persists.

COUNTERACTION OF THE INHIBITING EFFECTS OF VARIOUS SUBSTANCES ON NITELLA.

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I.

INTRODUCTION.

Previous observation[1,2] indicates that cresyl blue enters the vacuole of living cells of *Nitella* and *Valonia* much more rapidly in form of free base (predominating at higher pH values) than in form of salt (predominating at lower pH values), and that upon its entrance part of the free base is at once converted into salt depending on the condition of the sap (*viz.* pH value). Difference in the rate of penetration of these two forms of dye may be attributed to the difference in the extent to which they are taken up by each non-aqueous layer of protoplasm from one aqueous phase and given up to the other aqueous phase.

Any factors affecting the dye or the cell may alter the rate of penetration of dye into the vacuole. For example, the rate is altered by the effect of salt and hydrogen ions on the dye.

As to the control of the rate by the cell, it is uncertain just what part of the cell plays the predominant rôle. Cells of *Nitella* consist of cell wall surrounding a very thin layer of protoplasm enclosing a central vacuole filled with aqueous sap at about pH 5.5. If we neglect the cell wall by assuming that the effect of substances on the cell wall are insufficient in the present case to change the rate of penetration of dye into the vacuole, then we may imagine the penetration of dye as dependent on the diffusion of free base through the protoplasm into the vacuole. The protoplasm may be assumed to consist of an

[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561.

[2] Irwin, M., *J. Gen Physiol.*, 1926–27, x, 75.

(New York II) were employed. The condition of the cell during the experiment is very difficult to determine exactly. Reversible injury is almost impossible to detect. For this reason it was necessary to carry out experiments on a uniform basis, in order that the condition of the cells might be as uniform as possible. Cells of uniform size, thickness, and turgidity, from the central portion of the plant, were chosen. Cells collected near New York were kept in the laboratory not longer than 4 days after they were collected. Such concentrations of solutions as maintain the cells in as good a condition as possible during the experiments were selected. The criteria for the condition of cells were as follows: (1) turgidity tested by means of touch (as cells become injured they lose turgidity more and more until they collapse after death); (2) the ability of cells to live for several hours in the test solutions while the experiments lasted only a few minutes; (3) the behavior of the cells after experiments: cells were transferred from the test solutions to tap water, and after 1 day the turgidity and mortality of cells were compared with those of the control cells (cells which have not been exposed to any test solutions). Judged by these criteria, the cells referred to in these experiments seemed normal.

Unless otherwise stated the exposure of cells to different solutions other than the dye solution lasted 10 minutes, and such solutions were not stirred. Buffers were made up according to directions given by Clark.[9] For phosphate buffer, a suitable mixture of M/15 Na_2HPO_4 and M/15 KH_2PO_4 was diluted ten times; for borate buffer, a suitable mixture of M/5 boric acid and M/20 borax was diluted ten times. Brilliant cresyl blue (prewar Grübler) was used. If the dye solutions were stirred this was done at the rate of one revolution per minute.

The pH values of the solutions made up with buffer mixtures were determined by means of the hydrogen electrode. Those of other solutions were determined colorimetrically.

In the 7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture the proportion of the mixture used was 1 of M/20 borax to 9 of M/5 boric acid and this mixture was diluted ten times with distilled water, while in the dye made up with phosphate buffer mixture the proportion was 8.85 of M/15 secondary to 1.15 of M/15 primary phosphates and the mixture diluted ten times. In 35×10^{-5} M dye solution at pH 6.79 made up with phosphate buffer mixture the proportion used was 4 of M/15 secondary to 6 of the M/15 primary phosphates, and this mixture was diluted ten times with distilled water.

In every case cells were wiped, dipped in distilled water for 3 seconds, and wiped again before they were placed in any solution.

The pH value of the sap was determined colorimetrically using either methyl red or brom cresol purple as an indicator. The pH value of the sap remained unchanged in every experiment.

The concentration of dye in the sap was determined colorimetrically. The end

[9] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

TABLE I.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the solutions given in III, and are then placed in any of the dye solutions given in I. Buffer mixtures are given in Section II in the text.

I		II	III					
External dye solutions			Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid	Hydrochloric acid	Phosphoric acid
			pH 7.7	*pH 5.5*	*pH 4.8*	*pH 4.8*	*pH 4.2*	*pH 4.3*
When cells are placed for ½ minute in 7×10^{-6} M dye solution at pH 7.85 made up with borate buffer mixture	Dye stirred	Amount of dye in sap, $M \times 10^{6}$	21.1	9.7	19.2	16.2	15.2	12.8
		Percentage decrease or increase	Standard	54 per cent decrease	9 per cent decrease	23 per cent decrease	28 per cent decrease	39 per cent decrease
	Dye not stirred	Amount of dye in sap, $M \times 10^{6}$	12.4	5.0	9.5	9.0		
		Percentage decrease or increase	Standard	60 per cent decrease	23 per cent decrease	28 per cent decrease		
When cells are placed for ½ minute in 7×10^{-6} M dye solution at pH 7.85 made up with phosphate buffer mixture	Dye stirred	Amount of dye in sap, $M \times 10^{6}$	19.7	19.2	20.5	19.7		
		Percentage decrease or increase	Standard	2 per cent decrease	4 per cent increase	No change		
	Dye not stirred	Amount of dye in sap, $M \times 10^{6}$	11.4	11.7			11.0	12.4
		Percentage decrease or increase	Standard	3 per cent increase			3 per cent decrease	9 per cent increase
When cells are placed for 1 minute in 35×10^{-6} M dye solution at pH 6.79 made up with phosphate buffer mixture	Dye not stirred	Amount of dye in sap, $M \times 10^{6}$	19.7	15.2				
		Percentage decrease or increase	Standard	23 per cent decrease				

of the cell was cut, and the sap was gently squeezed out onto a glass slide. The sap was then drawn up into a capillary tube and the color of this tube was matched with that of the capillary tube containing a standard dye solution. The dye measured represents the total amount of dye found in the sap, consisting of uncombined free base and salt, and possibly some dye in combination with constituents of the sap.

The experiments were carried out in an incubator at 25 ± 0.5°C. During the experiment diffused light entered the incubator through small air holes and through a partly open door.

Each figure given in the tables represents an average from 80 to over 100 readings on individual cells and the probable error of the mean is less than 7 per cent of the mean.

III.

Removal of the Inhibiting Effect of Phosphate Buffer Mixture, Phosphoric Acid, and Hydrochloric Acid.

When cells were exposed for 10 minutes to hydrochloric acid, to phosphoric acid, or to phosphate buffer mixture, the pH value of the sap was found to remain unchanged. If such cells were placed in the dye solution made up with a borate buffer mixture at pH 7.85, the rate of penetration as compared to the control was decreased,[10] as shown in Table I.

On comparing the inhibiting effect of these substances it was found, as shown in Table I., that hydrochloric acid brought about the least and phosphate buffer mixture the greatest effect. For example, at both pH 4.2 and at pH 4.8, hydrochloric acid brought about less effect than phosphoric acid, while even at pH 5.5, where practically no effect of hydrochloric acid and only a slight effect of phosphoric acid might be expected, the phosphate buffer mixture brought about considerable inhibiting effect (Table I).

[10] These results confirm those already published by the writer. The percentage decrease given in the experiments described in the present paper, however, is a little higher, but the difference may be considered as due to experimental error or to differences in the cells used, *cf.* Table VI, *J. Gen. Physiol.*, 1926–27, x, 281. Cells in both these cases were collected in New York, and their behavior seemed different from those collected in Cambridge in that they were affected by the phosphoric acid and hydrochloric acid at pH 4.8 to about the same extent as the Cambridge *Nitella* were affected by these acids at pH 4 (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 56 and 57).

TABLE I.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the solutions given in III, and are then placed in any of the dye solutions given in I. Buffer mixtures are given in Section II in the text.

I		II	III					
External dye solutions			Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid	Hydrochloric acid	Phosphoric acid
			pH 7.7	pH 5.5	pH 4.8	pH 4.8	pH 4.2	pH 4.3
When cells are placed for ½ minute in 7×10^{-4} M dye solution at pH 7.85 made up with borate buffer mixture	Dye stirred	Amount of dye in sap, $M \times 10^5$	21.1	9.7	19.2	16.2	15.2	12.8
		Percentage decrease or increase	Standard	54 per cent decrease	9 per cent decrease	23 per cent decrease	28 per cent decrease	39 per cent decrease
	Dye not stirred	Amount of dye in sap, $M \times 10^5$	12.4	5.0	9.5	9.0		
		Percentage decrease or increase	Standard	60 per cent decrease	23 per cent decrease	28 per cent decrease		
When cells are placed for ½ minute in 7×10^{-4} M dye solution at pH 7.85 made up with phosphate buffer mixture	Dye stirred	Amount of dye in sap, $M \times 10^5$	19.7	19.2	20.5	19.7		
		Percentage decrease or increase	Standard	2 per cent decrease	4 per cent increase	No change		
	Dye not stirred	Amount of dye in sap, $M \times 10^5$	11.4	11 7			11.0	12.4
		Percentage decrease or increase	Standard	3 per cent increase			3 per cent decrease	9 per cent increase
When cells are placed for 1 minute in 35×10^{-4} M dye solution at pH 6.79 made up with phosphate buffer mixture	Dye not stirred	Amount of dye in sap, $M \times 10^5$	19.7	15.2				
		Percentage decrease or increase	Standard	23 per cent decrease				

From these results the following conclusion may be drawn.

1. The inhibiting effect of hydrochloric acid seems to be due chiefly to the action of hydrogen ions on the protoplasm since it has been already found by the writer that the chlorides[6] do not seem to bring about any marked inhibiting effect.

2. The greater effect of phosphoric acid may be due either to the specific effect of the anion or to greater penetration of the acid as an undissociated molecule and subsequent dissociation, thereby lowering the pH value of the protoplasm more than is the case with hydrochloric acid. With the lowering of the pH value of the protoplasm the concentration of free base is decreased. We might then expect a decrease in the rate of penetration of dye if we assume that the rate is controlled by the diffusion of free base from the protoplasm into the vacuole.

3. The inhibiting effect produced by phosphate buffer, on the other hand, cannot be attributed primarily to the presence of hydrogen ions and phosphoric anions in the solution, for its inhibiting effect, even when its pH value is 5.5, is greater than that of hydrochloric or phosphoric acid at pH 4.2 (*cf.* Table I). It must be attributed largely to the presence of monovalent base cations, such as sodium and potassium, which bring about an appreciable decrease in the rate of penetration of dye when cells are exposed to them in the same way.

When the dye solution was not stirred the extent of decrease in the rate of penetration of dye was slightly greater than when it was stirred as shown in Table I. It is uncertain whether the greater decrease brought about by lack of stirring is due to the less rapid diffusion of the substances from the cell or to the lowering of the pH value of the solution just outside the cell, as resulting from the accumulation of these substances as they diffuse out of the cell, or due to experimental error.

On the other hand, if cells were exposed to these various solutions and placed in the dye solution made up with phosphate buffer mixture at pH 7.85 the rate of penetration instead of decreasing as before was found to be about the same[7] as that in the case of the cells transferred directly from tap water to the same dye solution. This was the case whether the dye solution was stirred or not (Table I). This absence of the inhibiting effect was not chiefly due to the effect of the phosphate

buffer mixture alone on the protoplasm, because when cells were washed in the phosphate buffer mixture at pH 7.85 for ½ minute (stirred) before they were placed in the dye + borate at pH 7.85, the usual inhibiting effect was observed (Table III).

Unless the cells were first exposed to the inhibiting substances and then placed in the dye solutions there was no marked difference in the behavior of the dye + borate and of the dye + phosphate at pH 7.85. For example, if cells were transferred directly from tap water to these two dye solutions at pH 7.85 the rate of penetration of dye was about the same in dye + borate as in dye + phosphate (see control, Table I).

It would be desirable to know the cause of this difference in behavior. We might assume that the decrease in the rate of dye penetration with the dye + borate at pH 7.85 is due to the decrease in the pH value of the dye solution just outside the protoplasm, which might take place as the dye comes in contact with the substance to which the cells have been exposed before they were placed in the dye and which might adhere to the surface of the protoplasm, and that the decrease does not take place with the dye + phosphate at pH 7.85 because it is sufficiently buffered to keep the pH value constant. In so far as the dye + borate at pH 7.85 and dye + phosphate at pH 7.85 are concerned, this explanation may hold since the latter is more buffered than the former, but when we compare their behavior with that of the dye + phosphate at pH 6.79, we find this interpretation untenable. On this assumption we should expect no decrease in the rate of dye penetration with the dye + phosphate at pH 6.79, because it is even more buffered than the dye + phosphate at pH 7.85, but the experiments show that there is about 23 per cent decrease when cells are exposed, for example, to a phosphate buffer solution at pH 5.5 (*cf.* Table I). Thus we may conclude that the occurrence of a decrease with dye + borate at pH 7.85 and its absence with dye + phosphate at pH 7.85 cannot be attributed to the difference in the changes of pH values of the dye solutions just outside the protoplasm, as a result of the difference in their buffer effects.

A better assumption is that the dye is affected by the cations (sodium and potassium) present in these buffer solutions with which the dye is made up in such a way as to counteract the inhibiting effect of various substances on the protoplasm. According to this theory the

greater the concentration of such cations in the dye the less the inhibiting effect. This assumption is supported by the results which show that the counteraction of the inhibiting effect is proportional to the concentration of base cations such as sodium and potassium in the dye solutions, in the following order: dye + phosphate at pH 7.85 > dye + phosphate at pH 6.79 > dye + borate at pH 7.85 (Table I). If this supposition is correct an addition of the proper concentration of

TABLE II.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to any one of the solutions (not stirred) stated in III, and are then placed in one of the brilliant cresyl blue solutions (stirred) stated in I for ½ minute. Buffer mixtures are given in Section II in the text.

I	II	III			
External dye solution		Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid
			pH 5.5	*pH 4.2*	*pH 4.3*
7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture containing 0.01 M magnesium chloride	Amount of dye in sap, M $\times$ 10^5	22.8	22.8	21.4	22.1
	Percentage decrease or increase	Standard	No change	6 per cent decrease	3 per cent decrease
7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture containing 0.02 M sodium chloride	Amount of dye in sap, M $\times$ 10^5	24.2	22.4	22.4	22.8
	Percentage decrease or increase	Standard	7 per cent decrease	7 per cent decrease	6 per cent decrease

base cations to the dye + borate at pH 7.85 should cause the dye + borate at pH 7.85 to behave like the dye + phosphate at the same pH value and should counteract the inhibiting effect. The experimental results show that this is precisely what takes place. If cells previously exposed to an inhibiting substance were placed in the dye + borate solution containing sodium chloride the rate of penetration of dye remained the same[7] as that of the control (cells transferred directly from

the tap water to the same dye solution, as shown in Table II). That this counteraction of the inhibiting effect is not due to the effect of the sodium chloride on the borate buffer mixture alone is indicated

TABLE III.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the various solutions (not stirred) stated in III, are then washed for ½ minute in one of the solutions (stirred) stated in I, and are finally placed for ½ minute in 7 × 10^{-5} M brilliant cresyl blue solution (stirred) made up with borate buffer mixture at pH 7.85. Buffer mixtures are given in Section II in the text.

I	II	III			
Solution in which cells are washed		Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid
			pH 5.5	*pH 4.2*	*pH 4.3*
Borate buffer mixture at pH 7.85	Amount of dye in sap, M × 10^5	21.7	9.0	16.6	17.3
	Percentage decrease or increase	Standard	59 per cent decrease	24 per cent decrease	20 per cent decrease
Borate buffer mixture at pH 7.85 containing 0.02 M NaCl	Amount of dye in sap, M × 10^5	22.4	8.6	12.4	9.0
	Percentage decrease or increase	Standard	62 per cent decrease	45 per cent decrease	60 per cent decrease
Borate buffer mixture at pH 7.85 containing 0.01 M $MgCl_2$	Amount of dye in sap, M × 10^5	21.7	23.4	22.1	23.1
	Percentage decrease or increase	Standard	8 per cent increase	2 per cent increase	6 per cent increase
Phosphate buffer mixture at pH 7.85	Amount of dye in sap, M × 10^5	21.0	9.5	16.0	13.0
	Percentage decrease or increase	Standard	55 per cent decrease	24 per cent decrease	38 per cent decrease

by the following experiments. When cells previously exposed were washed for ½ minute in the borate buffer solution at pH 7.85 containing sodium chloride (stirred), before they were placed in the dye + borate solution containing no sodium chloride, the washing did not remove this inhibiting effect but, in fact, the rate of penetration of dye was found to decrease, even more than when the cells had not been washed (Table III).

Not only addition of monovalent base cations but addition of bivalent base cations to the dye + borate solution at pH 7.85 also removed the inhibiting effect (Table II). There was a difference, however, between the behavior of the monovalent and bivalent base cations, in that washing cells with the former just before the cells were placed in the dye increased the inhibiting effect while washing with the latter removed the inhibiting effect (Table III). This removal of the inhibiting effect in this case seems to be due chiefly to the magnesium chloride and only slightly due to the borate buffer mixture since washing the cells for ½ minute in borate buffer mixture at pH 7.85 (stirred) containing no magnesium chloride only partly removed the inhibiting effect of the hydrochloric acid and phosphoric acid and had practically no influence on the inhibiting effect of the phosphate buffer mixture at pH 5.5 (*cf.* Table III).

IV.

Removal of the Inhibiting Effect of Sodium Chloride.

If the assumption is correct that the inhibiting effect of the phosphate buffer mixture is chiefly due to the action of monovalent base cations on the protoplasm, and if it is this effect that is removed under the experimental conditions discussed in Section III, we should expect the inhibiting effect of sodium in sodium chloride to be likewise removed. When the experiments were made to test this point it was found that the inhibiting effect of sodium chloride manifested when the dye was made up with borate buffer solution at pH 7.85 was removed if cells previously exposed to 0.02 M solution of sodium chloride were treated in the following manner.

1. If cells were placed in the dye solution made up with phosphate buffer mixture at pH 7.85 (Table IV). The removal is not due to the

effect of phosphate buffer alone because when cells were washed in this buffer solution containing no dye the inhibiting effect was not removed (Table IV).

TABLE IV.

Comparison of the amount of dye in the vacuole of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the solutions (not stirred) stated in III and are then placed for 1 minute in one of the 7×10^{-5} M brilliant cresyl blue made up with solutions (not stirred) stated in I. Buffer mixtures are given in Section II in the text.

I	II	III			
External dye solution		Tap water or control	NaCl	$MgCl_2$	0.02 M NaCl + 0.01 M $MgCl_2$
			0.02 M	*0.01 M*	
Made up with borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	18.6	7.6	20.7	16.9
	Percentage decrease or increase	Standard	59 per cent decrease	11 per cent increase	9 per cent decrease
Made up with phosphate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	19.0	20.0		
	Percentage decrease or increase	Standard	5 per cent increase		
Cells are washed for 1 minute in phosphate buffer mixture at pH 7.85 before they are placed in the dye solution made up with borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	18.7	8.3		
	Percentage decrease or increase	Standard	56 per cent decrease		
Cells are placed in borate buffer mixture at pH 7.85 containing 0.01 M $MgCl_2$	Amount of dye in sap, $M \times 10^5$	19	18.8		
	Percentage decrease or increase	Standard	1 per cent decrease		

2. If cells were placed in dye solution containing sodium chloride[6] or magnesium chloride (Table IV).

3. If cells were washed with bivalent or trivalent cations[6] just before the cells were placed in the dye.

A salt, such as magnesium chloride, may remove the inhibiting effect in absence[5] of dye but phosphate buffer mixture is capable of removing it only in presence of dye (Table IV).

Thus we may conclude that the inhibiting effect of sodium chloride may be removed just as is the case with that of the phosphate buffer mixture.

In addition, it was found that when cells were exposed to a mixture of 0.01 M magnesium chloride and 0.02 M sodium chloride, before they were placed in the dye + borate solution at pH 7.85, the inhibiting effect of sodium was practically removed (Table IV).

As in the case of the other substances discussed in Section III, we may also assume in the case of sodium chloride that the inhibiting effect of sodium on the protoplasm is counteracted by the presence of base cations, such as sodium, potassium, and magnesium, in the dye solution, or by magnesium in absence of dye. It is difficult to state just how this takes place: the base cations above mentioned may act on the dye in such a way that dye is altered so as to be able (1) to penetrate more readily the protoplasm which had been already affected by sodium chloride or (2) to drive the sodium out of the protoplasm or (3) to affect the protoplasm in such a way as to nullify the inhibiting effect of sodium.

Regarding the first assumption the following may be stated. We do not know whether sodium chloride, for example, affects the dye in such a way as to increase its power to penetrate to the extent of counteracting entirely the inhibiting effect of sodium chloride, though experiments[5] have shown that when the dye contains sodium chloride it penetrates more rapidly into the cells and that this increase is a little greater when the cells have been previously exposed to sodium chloride. Spectrophotometric analysis of the dye shows that sodium chloride at 0.1 M does not affect the dye sufficiently for the change to be detected by this method but at higher concentrations it affects the dye in such a way that the absorption curve resembles that of a higher concentration of dye.

As to the second assumption, the experimental results so far obtained are not sufficiently complete to be reported but the writer hopes to be able to present them later.

In relation to the third assumption previous experiments[6] have shown that an inhibiting effect was brought about whether the cells were exposed to the sodium chloride in presence or absence of dye (during this period no dye entered the vacuole) so long as they were subsequently placed in the dye borate containing no sodium chloride at pH 7.85, but this effect was removed if the latter dye solution contained sodium chloride. These results seem to suggest that the dye at such a concentration is incapable of very readily counteracting the inhibiting effect of sodium chloride on the protoplasm but that it is affected by sodium chloride in such a way as to penetrate the protoplasm more readily. A detailed account of these experiments will be subsequently published.

V.

Removal of Inhibiting Effect of Sodium Borate.

In previous sections we have found that the inhibiting effects of phosphate buffer mixture, sodium chloride, hydrochloric acid, and phosphoric acid may be removed. It seems that the counteraction of this inhibiting effect is dependent on the counteraction of the cation effects and that anions do not play an important rôle in this respect. It might be of interest to see if salts and acids with cations common to those already studied, but with different anions, might give some other result. For this purpose sodium borate and boric acid were used.

As shown in Table V, 0.005 M sodium borate (containing 0.01 M sodium), and borate buffer mixture[11] at pH 8.4 (containing 0.0015 M sodium), showed an inhibiting effect while boric acid did not; the cells were first exposed to these solutions and then placed in dye solution at pH 7.85 made up with borate buffer mixture. The inhibiting effect with sodium borate was found to be about 38 per cent, while that of the borate buffer mixture at pH 8.4 was about 22 per cent. The

[11] The borate buffer mixture at pH 8.4 was made up with a proportion of three of M/20 borax to seven of M/5 boric acid and diluted ten times.

TABLE V.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when they are first exposed for 10 minutes to one of the solutions stated in III, and are then placed for ½ minute in 14×10^{-5} M brilliant cresyl blue solution (stirred) at pH 7.85 as stated in I. Buffer mixtures are given in Section II in the text.

I	II	III				
External dye solution		Tap water or control	Boric acid	Borate buffer mixture	Sodium borate	Sodium chloride
			0.01 M	*pH 8.4*	*0.005 M*	*0.01 M*
Made up with borate buffer mixture	Amount of dye in sap, $M \times 10^5$	41.1	38.7	31.7	25.5	16.9
	Percentage decrease or increase	Standard	6 per cent decrease	23 per cent decrease	38 per cent decrease	59 per cent decrease
Made up with phosphate buffer mixture	Amount of dye in sap, $M \times 10^5$	39.0	42.8	42.4	43.5	41.6
	Percentage decrease or increase	Standard	10 per cent increase	9 per cent increase	12 per cent increase	7 per cent increase
When cells are washed for ½ minute in phosphate buffer mixture at pH 7.85 before they are placed in the dye made up with borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	41.0			29.7	
	Percentage decrease or increase	Standard			28 per cent decrease	

absence of the inhibiting effect with boric acid might be explained on the basis that though it enters the protoplasm as an undissociated molecule it is too weak an acid to lower the pH value of the protoplasm sufficiently to bring about any inhibiting effect, or on the basis of the specificity of the anions.

In view of the fact that boric acid gives no inhibiting effect and that there is greater inhibiting effect the higher the concentration of sodium in the borate solution, we may conclude that the inhibiting effect of the borate buffer mixture is brought about chiefly by the action of sodium on the protoplasm and not by the borate ions. But since sodium borate, and sodium chloride, containing approximately the same concentration of sodium do not bring about an inhibiting effect to the same extent, we may suggest that other ions must also play some part either in counteracting or accelerating the effect of these cations.

The inhibiting effect of sodium borate, just like that of other substances described in Sections III and IV, was found to be removed if cells previously exposed were placed in the dye + phosphate solution at pH 7.85, but merely washing the cells with phosphate buffer solution at pH 7.85 did not remove this effect to any appreciable extent (Table V).

CONCLUSION.

It is assumed that (1) the inhibiting effect of hydrochloric acid is due to the action of hydrogen ions on the protoplasm, (2) the inhibiting effect of phosphoric acid (at the pH value in which hydrogen ions have practically no effect) is due to either a specific effect of the phosphate ions on the protoplasm or due to the entrance of the acid as undissociated molecule and its lowering of the pH value of the protoplasm by its subsequent dissociation, (3) the inhibiting effect of sodium chloride and sodium borate is due to the action of sodium on the protoplasm, (4) the inhibiting effect of phosphate buffer mixture is due primarily to the action of sodium and potassium ions and partly to the action of phosphoric acid on the protoplasm (see Section III).

The inhibiting effect is counteracted when cells previously exposed to these inhibiting substances are placed in the dye solution containing certain concentration of base cations or washed in a salt solution containing bivalent cations. It is not certain whether this removal of the

inhibiting effect in the presence of dye is due primarily to the action of these cations on the dye altering its nature so that it can penetrate the cell more readily or enabling it to nullify the inhibiting effect of these substances on the protoplasm either by displacing them from the protoplasm or by increasing the general permeability of the protoplasm.

It is uncertain as to whether the effect of the ions on the protoplasm is at the surface or in the interior. In case the ions penetrate, this will not necessarily contradict the theory (presented in Section I) stating that the outer layer of protoplasm is non-aqueous.[12] Certain non-aqueous substances absorb ions to a certain extent, and it may very well be that only a very small amount of ions penetrating into the protoplasm is sufficient to bring about an inhibiting effect.

The inhibiting effect discussed here does not indicate that the protoplasm is altered to decrease the rate of penetration of all substances. These experiments apply only to the penetration of brilliant cresyl blue.

SUMMARY.

When living cells of *Nitella* are first exposed to (1) phosphate buffer mixture, or (2) phosphoric acid, or (3) hydrochloric acid, or (4) sodium chloride, or (5) sodium borate, and are then placed in a solution of brilliant cresyl blue made up with a borate buffer mixture at pH 7.85, the rate of penetration of the dye into the vacuole is decreased as compared with the rate in the case of cells transferred directly from tap water to the same dye solution.

When cells exposed to any one of these solutions are placed in the dye solution made up with phosphate buffer solution at pH 7.85, the rate of penetration of dye into the vacuole is the same as the rate in the case of cells transferred from the tap water to the same dye solution.

It is probable that this removal of the inhibiting effect is due primarily to the presence of certain concentration of sodium and potassium ions in the phosphate buffer solution. If a sufficient concentra-

[12] Overton claimed that substances soluble in lipoid penetrated living cells (Overton, E., *Jahrb. wissen.ch. Bot.*, 1900, xxxiv, 669).

tion of sodium ions is added to the dye made up with a borate buffer mixture the inhibiting effect is removed just as it is in the case of the dye made up with the phosphate buffer mixture.

The inhibiting effect of some of these substances is found to be removed by the dye containing a sufficient concentration of bivalent cations, or by washing the cells with salts of bivalent cations.

The inhibiting effect and its removal are discussed from a theoretical standpoint.

GEOTROPIC ORIENTATION OF YOUNG MICE.

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I.

The upward geotropic orientation of young rats can be described by simple mathematical expressions (Crozier and Pincus, 1926, 1926–27, *a*, *b;* Pincus, 1926–27). In essence these formulæ also apply precisely for the geotropism of invertebrates (Wolf, 1926–27; Crozier and Stier, 1927–28; Wolf and Crozier, 1927–28). It seemed important to repeat with young mice the initial experiments with rats (Crozier and Pincus, 1926–27, *a*), partly as an independent test of the general results and in further part to investigate objectively certain sources of variation inherent in data obtained as averages from observations upon numerous individuals.

Young mice proved to be not such good material for these experiments as the rats. Their creeping is more irregular, interrupted. The stock used was not of a genetic homogeneity comparable to that of the rats (*loc. cit.*), having been inbred for but four generations. The extent of average upward orientation on an inclined plane is nevertheless found to be a definite function of the active gravitational component, and the results are, in fact, closely comparable to those earlier secured.

II.

Members of three successive litters (seven individuals) from the same parents were tested from 9 days after birth until the eyes opened. They were allowed to creep upon an inclined board on which was a tightly stretched, close wire mesh, to afford good footing. They were at first observed under red light. In preliminary tests the paths taken were recorded by chalk on the wire mesh and the angle of upward orientation (θ) was measured with a protractor. The mice were dark-adapted before readings were taken.

It was found that the mice would generally move for only short distances before "hesitating" and swinging about a great deal, then continuing again. It was thought that the heat from the red lantern, or the red rays, might disturb the geotropism, and they were accordingly observed in darkness. Small dots of luminous paint were placed in a line on the back of each animal. Sheets of paper, on which were ruled horizontal lines, were pinned at one side of the board. The movements of the mice were recorded with pencil by placing a ruler on the paper parallel to the path of the animal. The ruler had a very slightly luminous edge, which was kept shielded from the mouse. Every upward movement of the mouse of more than about 5 cm. was recorded. The sequence in which the trails were recorded was noted by numbering each path on the paper. From fifteen to thirty readings were taken at a time with each mouse. Where the mouse was brought back to the bottom of the board a new trail was begun. One run up the length of the board would give from two to five readings. The mice were started at the bottom of the board, each time headed downward. The purpose of this was to try to determine any general or progressive sequence in variation of orientation. Record was kept of the ages and hours of experimentation for each individual mouse. Detailed study of the orientation angles as obtained in successive tests showed no systematic effect of fatigue, growth, or training. Values of θ gotten by the second method were found to agree well with those secured in the initial experiments under red light. Variability of response seemed definitely increased just before the opening of the eyes.

III.

Over 800 readings were taken with mice from one cage. The results are summarized in Table I. Below an inclination of the board of 20° (α) there was no definite upward orientation. Above $\alpha = 20°$ practically all movements were upward. The tilt was gradually increased to 70°. Above 50° there seemed to be no further upward orientation. The precision of orientation, inversely expressed by the probable error, increased with α.

The average angle of orientation, θ, as an empirical relationship, is directly proportional to the logarithm of the sine of the angle of the creeping plane:

$$\theta = k \log \sin + C$$

This relationship is shown in Fig. 1, the points falling almost on a straight line, within the limits of ± 3 times the probable errors of the means, except that above 50° there is no further upward orientation of the mice.

The clarity of this relationship is not so great as with the rats (Crozier and Pincus, 1926–27, *a*, *b*), which is undoubtedly due to the lesser uniformity of the material and to the inhomogeneity of the averages resulting from the inclusion of readings from a number of individuals at different times. As was previously pointed out, the

TABLE I.

Mean angles of upward orientation (θ) of seven young mice upon a plane inclined at angle α to the horizontal. In the last column, the P.E. of θ, from the first forty observations at each magnitude of α, is given as a percentage of the corresponding mean θ.

α	No. of observations	θ	$\frac{\text{P.E}_{\theta.40} \times 100}{\theta}$
			per cent
20°	68	52.15 ± 1.85°	4.44
25°	115	58.72 ± 1.29°	3.72
30°	128	65.55 ± 0.93°	2.68
35°	173	68.63 ± 0.84°	1.59
40°	83	79.22 ± 0.58°	0.998
45°	59	82.31 ± 0.51°	0.697
50°	74	83.71 ± 0.37°	0.636
55°	62	83.07 ± 0.49°	0.784
60°	49	81.48 ± 0.67°	0.852
70°	46	83.62 ± 0.41°	0.897

mice do not offer intrinsically as good material. They were not from as uniform a genetic strain, the mode of progression is not so favorable, and data from a number of individuals (seven) were averaged together. Indeed, it was found that a larger number of qualitatively similar readings from later experiments with mice of the same strain, but not litter mates, could be averaged in without materially affecting the results. Furthermore, every movement of the mice was recorded. It is obvious to the observer that at times certain outside influences tend to throw the mouse off its path; slipping on the board,

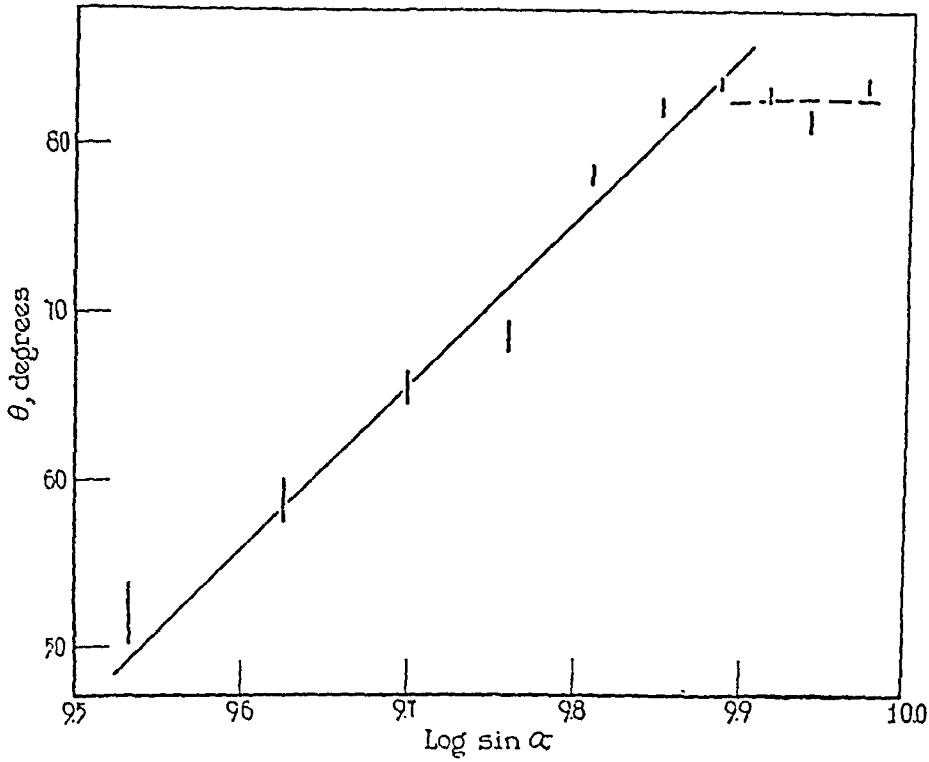

FIG. 1. Between 20° and 50° inclination of the plane of creeping the limitation of upward orientation of young mice is proportional to the logarithm of the gravitational component. Data (827 readings) in Table I (see text). The plotted bars are centered on the means and the vertical extent = 2 P.E.

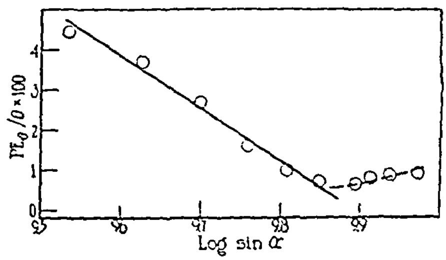

FIG. 2. The precision of orientation, as measured by the proportionate scatter of the readings, increases in proportion to log sin α, up to 50°. At higher inclinations mechanical difficulties impede precise orientation, and the variability increases.

running into an obstacle, and the like. Hence the variability of orientation was increased by not discounting certain runs. The precision of the measured angles of orientation may be estimated by the coefficients of variation, taken as the probable errors of the means for the first forty readings expressed as percentages of the means. The P.E. of the mean θ decreased steadily as α increases. The values of $\text{P.E.}_m/m \times 100$ decrease linearly with log sin α, as found with the

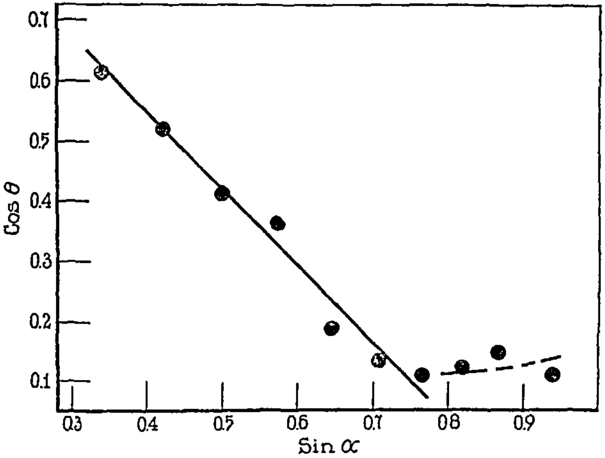

FIG. 3. As previously found for rats, cos θ decreases linearly with increase of sin α, up to 50°.

rats. Above $\alpha = 50°$ the angle θ exhibits increased variability, absolutely and proportionately. This is presumably due in large part to mechanical difficulties in maintaining foothold, such that the average value of θ seems not to be increased above $\alpha = 50°$ (*cf.* Fig. 2).

Again, as with the rats, *cos θ* decreases linearly as *sin α* increases. This is made evident in Fig. 3.

SUMMARY.

The geotropic orientation of young mice, on a plane at angles between 20° and 50° to the horizontal, obeys the equations previously found for young rats by Crozier and Pincus (1926–27). When the individuals tested in such experiments are not of the utmost uniformity, the variability of the measured orientations is increased.

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STUDIES ON THE PERMEABILITY OF MEMBRANES.

IV. Variations of Transfer Numbers with the Dried Collodion Membrane Produced by the Electric Current.

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In previous papers of this series (1, 2) we have discussed two different methods for determining the transfer number of electrolytes with the narrow pore type of collodion membrane. The first consisted in measuring the P.D. between two electrolyte solutions in a constant ratio of concentration (1:2) in different ranges of concentration. In the second the transfer numbers were calculated directly from electric transfer experiments. It was shown that in general the two methods led to the same result, namely that the transfer number of the anion is always smaller in the membrane than it is in free aqueous solution and that the magnitude of this transfer number largely depends on the concentration. Nevertheless there was a slight but evident discrepancy between the values obtained by the two methods. We also pointed out that the magnitude of the transfer numbers changed a little when several electric transfer experiments were performed in immediate succession with the same membrane. As belief in the validity of the fundamental assumptions is weakened by the presence of unexplained discrepancies, a further investigation of these phenomena was undertaken.

Description of Experiments.

The technique employed in carrying out this series of experiments was essentially the same as has been previously described. The solutions in the anode and cathode compartments were always KNO_3 and KCl respectively. Analyses concerned only the Cl entering the KNO_3 solution.

Two series of experiments were performed. The first series concerned itself with a study of what we shall refer to as a polarization effect. We had previously observed that when a membrane which for several days had been in contact with distilled water was used for two successive experiments the transfer number for Cl obtained by the second experiment was slightly lower than that obtained by the first. The experiments of this series were designed so as to exaggerate this effect as much as possible. Two experiments were always performed successively in which the current strength and length of time were identical (4 milliamperes for 15 minutes) but between the two experiments the membrane was thoroughly polarized by introducing fresh solutions and passing a current of relatively high intensity for several minutes (usually 0.15 amperes for 2 minutes). The experiments were carried out with two membranes of the flat type in 0.5 N, 0.2 N, 0.1 N, 0.05 N, and 0.02 N concentrations.

TABLE I.

Closeness of Agreement between Total Ionic Transfers as Determined by Calculation from the Time and Current Intensity and Directly by Means of an Iodine Coulometer.

Experiment No.	Current intensity	Time	Total transfer by calculation	Total transfer by coulometer
	milliamperes	*min.*	*millimols*	*millimols*
123	2.0	30	0.0373	0.0378
129	2.0	30	0.0373	0.0403
130	2.0	40	0.0498	0.0504
191	4.0	15	0.0373	0.0378
230	0.5	120	0.0373	0.0380
231	0.5	120	0.0373	0.0373

The second series of experiments was planned so that the effect of varying the current intensity could be studied. In order to eliminate the effect of previous polarization just discussed the experiments of this series were carried out only after considerable polarization had been already established. This was accomplished by performing a third experiment after the completion of the two experiments described in the preceding paragraph. In this experiment the total quantity of current expressed in coulombs was approximately the same as in the

preceding experiments but the current intensity was higher (0.3 amperes) and the duration of the experiments quite brief (10 to 15 seconds). The results obtained by this experiment were then compared with those secured from the second experiment of the first series.

In the first series of experiments the total quantity of current was determined by the method described in the third paper of this series. This consisted in maintaining a current of constant intensity by means of a sensitive milliammeter regulated with a variable resistance and accurately observing the time of application. From this data the total amount of ionic transfer was computed from the formula

$$1 \text{ milliampere} \times 1 \text{ minute} = 0.000622 \text{ milliequivalents of ions.}$$

In the experiments of the second series where the duration was reduced to a few seconds it was not practical to maintain an accurately constant current intensity, especially as the more sensitive milliammeter had to be replaced by a simple ammeter. For this reason a coulometer was used to determine the total amount of ionic transfer.[1] The accuracy of the coulometer was tested by employing it in a number of the experiments of longer duration in which the current intensity and time were easily measured. The results obtained by the two methods were in excellent agreement (Table I).

DISCUSSION.

We may first consider in how far the transfer number calculated from the concentration chain method has agreed with the transfer

[1] An iodine coulometer of the Herroun type as described in Ostwald and Luther's text-book (3) was employed. The arrangement was slightly modified as we found difficulty in obtaining potassium iodide which was free of the iodate. The quantity of KI to be placed in the coulometer was accurately weighed and an identical quantity placed in a similarly shaped tube at the same time and covered with the same solution used in the coulometer. At the conclusion of the experiment the solutions from both tubes were titrated as quickly as possible and the difference between the two titrations taken as the correct result. Furthermore to lessen the amount of spontaneous oxidation of KI the highly acid HCl solution used by Herroun was replaced by a 2 per cent solution of KH_2PO_4 previously boiled to expel all traces of oxygen.

number observed in direct transfer experiments. In the third paper of this series (2) we published a graph[2] in which the chlorine transfer numbers with KCl as determined by the concentration chain method were compared with the results obtained in twenty-nine electric transfer experiments with the same membrane. Reference to this graph shows that in the more concentrated solutions the observed transfer number was always lower than the calculated. In the lowest

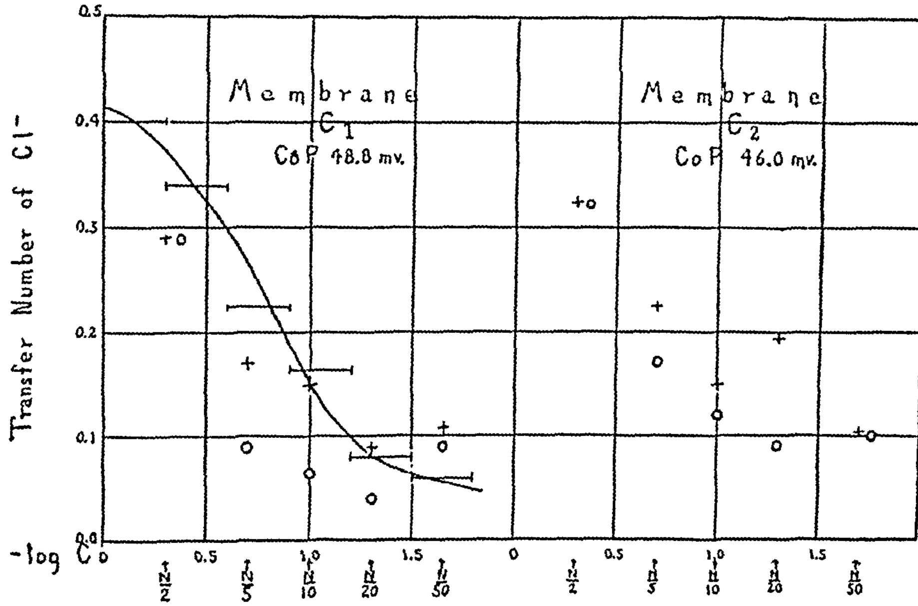

FIG. 1. Showing experimental results in first series of experiments performed with two membranes of the flat type. The transfer number for Cl yielded by the first experiment of each series is represented by +; the transfer number obtained from the second experiment by 0. For purposes of comparison the transfer number curve for the membrane C_1 calculated by the concentration chain method has been included.

concentrations the opposite was true. From the present series of experiments it becomes apparent that the observed transfer number rather than being a definite figure depends on the history of the experiment. When in a series of experiments with the same membrane the current intensity and duration of each experiment are kept constant and when several days have been allowed to elapse between experiments, during which time the membrane has been kept in dis-

[2] Michaelis, Weech, and Yamatori (2), Fig. 1, p. 700.

tilled water, then the transfer numbers observed are in close agreement. But when the time between successive experiments is short, *i.e.* some minutes, as in the experiments of our first series, the second transfer number is often different from the first. This indicates that some kind of polarization remaining from the first experiment has affected the result of the second and suggests that the result even in the first experiment has been more or less disturbed by the same polarization.

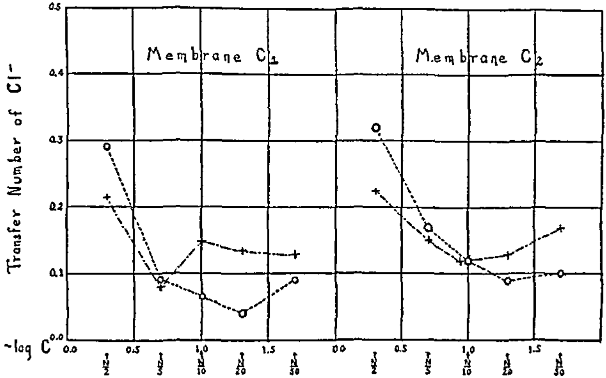

FIG. 2. Showing experimental results in second series of experiments performed with two membranes of the flat type. The transfer number for Cl yielded by the first experiment of each series (low amperage) is represented by 0; the transfer number obtained from the second (high amperage) by +. In all experiments the total quantity of current (coulombs) was approximately the same.

The direction and magnitude of this polarization disturbance is brought out by the experiments of our first series, the results of which have been summarized in Fig. 1. Here the transfer number of Cl has been charted against the logarithmic scale of the concentrations. The figures on the left-hand side of the chart concern one membrane, namely the one with which the graph previously published and mentioned above was prepared. For convenience of comparison the transfer number curve calculated by the concentration chain method

has been included in this chart. The figures on the right-hand side of the chart were obtained with another membrane in which the transfer numbers have not been obtained by the concentration chain method. Both membranes have given essentially the same result. It will be seen that in the experiments of high and of low concentrations, *i.e.* 0.5 N and 0.02 N, there is almost no difference in the transfer numbers obtained from the first and second experiments of the series. In the middle range of concentration there is a considerable difference between the results, the second experiment always yielding the lower transfer number.

In our second series of experiments, the results of which have been summarized in Fig. 2, a study was made of the effect on the transfer number of varying the rate at which the same quantity of electricity was passed through a membrane. Here it can be seen that in the more concentrated solutions (0.5 N) a current of high intensity has always yielded a lower transfer number than one of low intensity, that in dilute solutions the reverse is true, and that a middle range of concentration exists where currents of either high or low intensity give essentially the same transfer number.

An attempt to understand the nature of these two effects has led us to the following considerations. All available data have tended to establish the fact that in our membrane the mobility of Cl is less than that of K and that only the *degree* of the difference is modified by the conditions. Admitting the truth of this fact it follows that the passage of an electric current must be accompanied by a concentration of KCl on the cathode side of the membrane pores and a dilution on the anode side.[3] This phenomenon is merely a restatement of the facts

[3] As a demonstration of the existence of such a surface boundary concentration would provide a further confirmation of the fundament of this theory we thought it worth while to attempt to show this concentration by experiment. This was accomplished by utilizing a form of membrane similar to our flat bell jar type but in which the collodion was fastened to the end of a straight tube 1 inch in diameter. This form offers less opportunity for mechanical mixing of the different layers of the inside solution. Potassium chloride solutions of equal concentrations were placed on both sides of the membrane and an electric current passed by means of platinum electrodes placed at some distance from the membrane. The inside electrode was always the cathode. At the beginning of the experiment a 1 cc. pipette was placed within the tube and its tip allowed to rest against the membrane.

established by Hittorf, which early led to the concept of a "transfer number." However in our membrane an additional factor has been introduced with which Hittorf did not have to contend. In Hittorf's experiments the transfer number was almost independent of the concentration and hence of changes in concentration brought about by a current. In our experiments the transfer number being a function of the concentration may be altered by changes in concentration resulting only from the current. As the Hittorf effect tends to raise the concentration on one side of the membrane and decrease it on the other it follows that different transfer numbers will obtain in different parts of the membrane. Our analytical results based on Cl determinations concern themselves only with that transfer number holding for the anodic end of the membrane pores. In order to interpret them we must show the effect of the current itself on the concentration at this border.

TABLE II.

Demonstration by Chemical Analysis of the Change in Concentration at One Surface of the Collodion Membrane during the Passage of an Electric Current.

Co P of membrane	Approximate concentration of original KCl solutions	Current intensity	Time	Final Cl concentrations	
				Layer in contact with membrame	Solution distant from membrane
mv.	*M*	*milliamperes*	*min.*	*M*	*M*
49.7	0.01	1	30	0.0114	0.0098
50.0	0.01	2	30	0.0111	0.0092
48.3	0.01	2	30	0.0106	0.0091
32.7	0.02	5	20	0.0202	0 0189

After a suitable time the pipette was filled without stirring in order that as much as possible of the solution in direct contact with the membrane might be secured. Immediately thereafter a similar sample was taken from the upper layers of the solution and the two samples titrated for their content of Cl. It is evident that the area of greatest concentration brought about by the electric current must be a film of microscopic thickness coating the membrane surface. By removing an entire cc. merely from this region we did not hope to show the maximal concentration. Nevertheless the results obtained were quite sufficient to support the theory. The solution in contact with the membrane was always the more concentrated (Table II). With the analytical method for determining Cl used the maximum titration error is not above 0.04 cc. of N/100 $AgNO_3$ solution or 0.0004 millimols.

The primary effect of the electric current is to lower the concentration at this border. Inasmuch as the concentration here is lower than that originally present the transfer number of the Cl will be lower than would have been expected without polarization. It is likewise evident that the magnitude of this lowering will be greatest in the concentration range where the transfer number is most dependent on the concentration, *i.e.* the middle concentration ranges. In high and in low concentration where the slope of the curve expressing the dependence of the transfer number on concentration is slight the magnitude of this lowering must be minimal. This is in accordance with the observed facts. When a second experiment is performed immediately after a first one in order that polarization may be already established when the second experiment is started then it is observed that the figure obtained from the second experiment is still lower than that secured from the first in the middle ranges of concentrations and in the high and low concentrations essentially no difference between the two experiments can be discovered.

But it is necessary also to consider the change going on at the cathodic border. Here an increase in concentration is being brought about. This increase results in an increase in the transfer number of Cl, so that more KCl can enter the membrane than previously and gradually, as a result of the mutual effect of concentration and transfer number on each other, the whole concentration of KCl within the membrane is increased. Finally this increase will extend even to the anodic border. We shall refer to this as a "secondary effect." At the same time the force of the electric current tending to raise the concentration throughout the membrane is opposed by the force of spontaneous diffusion tending to equalize it throughout the whole system. As a result a stationary condition is reached as long as the current is maintained at constant intensity. Any change in the intensity of the applied current will, however, alter the level of this stationary condition. Inasmuch as this stationary condition may also extend through the membrane even to the anodic border, any change in its level will also alter the transfer number of Cl. To a certain extent the effect will be present even with currents of low intensity but with currents of high intensity it will be greatly exaggerated. Furthermore, if, as in our experiments, the transfer

number obtained with a current of low intensity is contrasted with that given by a current of high intensity, the change in transfer number whether greater or less will indicate the direction of alteration from the transfer number present in the absence of a current. That is, an arrow on the graph shown in Fig. 2 pointing from the result of the weak current experiment toward that of the strong current experiment will indicate the direction of the deviation. Extending this arrow in the opposite direction will indicate the transfer number holding under the initial condition.

Thus we have two contrary effects tending to change the concentration at the anodic border in opposite directions. We regret that we have been unable to form a mathematical representation of the equilibrium resulting from the two forces. We have therefore limited ourselves to some qualitative considerations suitable for an understanding of the total effect.

Thus far we have been talking about changes in the transfer number resulting from simple alterations in concentration. But it must be remembered that the degree of such changes depends greatly on the particular concentration. The rate of change of transfer number with concentration changes is expressed by the slope of the curve indicating the dependence of the transfer number on concentration and is very rapid in the middle ranges of concentration and very gradual in both high and low concentrations.

The combined effect of the two partial effects just discussed which begin at the two borders of the membrane may be expected to be dependent on the particular concentration under consideration.

When an experiment is being carried out in high concentrations there can be no appreciable change in transfer number at the cathodic border where the concentration is increased as further increases in concentration have very little effect on the transfer number. If the current is of low intensity the decrease in concentration at the anodic border will likewise be slight. If this decrease is sufficient to bring the concentration into the range where the transfer number is greatly dependent on concentration the transfer number will be lowered. To a certain extent this lowering may be apparent with currents of low intensity but with currents of high intensity it will be greatly exaggerated. In fact in our experiments in high concentrations the ob-

tained transfer numbers were always somewhat lower than the calculated; when the current was of high intensity the lowering was excessive. Furthermore in the experiments of our first series we obtained no difference in the results between a first and second experiment when an attempt was made to thoroughly polarize the membrane by the intermediate passage of a strong current. This may be explained by the fact that in high concentrations no essential change in transfer number results from the increase in concentration brought about at the cathodic border and there are no gradations of transfer numbers extending throughout the membrane. As no new stationary condition is established within the interior of the membrane a second experiment must give the same result as the first regardless of the intermediate treatment. The changes brought about at the surface layers of the membrane in contact with the fluids and upon which the change in transfer number when the current intensity is greatly increased depends is almost immediately reversible with alterations in the intensity of the applied current. Only changes involving the deeper layers of the membrane can persist any appreciable time after the current has been stopped and only such changes can exert an influence on succeeding experiments.

By a similar process of reasoning it can be shown that the changes going on in the middle range of concentration may lead to lower results than those obtained by a non-electric method. In this range any slight fluctuation in concentration will have a strong effect on the dependent transfer number. In general the primary effect at the anodic border will be greater than the secondary effect beginning at the cathodic border inasmuch as we are measuring the transfer number at this border. That is, when two successive experiments are performed with currents of the same intensity the second experiment will yield the lower result. Sufficient time has elapsed to allow the setting up of the stationary condition within the membrane and the result measured at the anodic border where the concentration becomes lower as the stationary condition is established is to lower the transfer number. The effect of varying the current intensity will be uncertain as the opposing forces acting on the transfer number at the two borders of the membrane are both maximal. In just one concentration the effect of the new stationary condition established by the

stronger current on the transfer number will be obscured because the two forces have neutralized each other. Any variation from this one concentration will cause either a lowering or a raising of the transfer number as one or the other of the two forces becomes more important. The agreement of this theory with the obtained results is evident from inspection of Figs. 1 and 2.

When the concentration under consideration is low it is evident that the decrease in concentration brought about at the anodic border will have little effect on the transfer number and the effects produced at the cathodic border even though secondary will predominate. In our experiments we were not able to show any appreciable difference between successive experiments provided there was no alteration of amperage but when the intensity was increased the transfer numbers became greater. In all experiments the obtained transfer numbers were greater than the calculated.

Thus, even though we have not been able to offer a mathematical presentation of the forces concerned in this series of experiments, it has nevertheless been possible to explain the results in a reasonable way.

SUMMARY AND CONCLUSIONS.

The transfer number of Cl in a KCl solution within the pores of a dried collodion membrane is always lower than 0.5. It depends on the concentration of the solution and decreases in general with decreasing concentration. However, the transfer number for any given KCl concentration has the significance of a definite and constant figure only when an infinitely small amount of coulombs is allowed to pass through the system. For finite durations of electric transfer experiments a polarization effect will always change the original transfer number. This polarization consists in an accumulation of the salt at the one boundary and a diminution at the other boundary of the membrane. Again, as the transfer number strongly depends on concentration, this change in concentration will bring about in its turn a gradual change in the transfer number too. It is shown under what conditions the transfer numbers for the anion as obtained by electic transfer experiments are higher or lower than the ones expected without polarization effect. Thus, by changing the character and magnitude of the force driving the ions across the membranes, and

according to the history of previous treatment of the membrane, the whole character of what we may call the specific permeablity for ions of the membrane may be varied without any substantial change of the membrane itself concerning its structure, its chemical composition, or its pore size.

Contemplation of the results obtained in this series of experiments in the light of the theoretical considerations just outlined has impressed us with the fallacy of speaking of the definite permeability of any type of membrane for electrolytes. The behavior of the membrane toward the passage of electrolytes depends on a variety of conditions. It may be recalled that different investigators have reported widely varying results concerning the permeability of certain physiological membranes for electrolytes. Such experiments as have been described in this paper may lead to an understanding of some of the factors responsible for such variations. We are aware that the collodion membrane in its simplicity is scarcely comparable to the extremely complicated biological membranes. Nevertheless any attempts to understand better the behavior of biological membranes may wisely begin with a study of the simplest prototypes.

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THE EFFECTS OF POLARIZATION UPON THE STEEL WIRE-NITRIC ACID MODEL OF NERVE ACTIVITY.

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In this series of experiments steel wires in a bath of 65 per cent nitric acid have been submitted to polarization by somewhat the same technique as that applied to nerve (Bishop and Erlanger, 1926), in the hope of further elucidating both the functioning of nerve and of the metastable steel-acid system itself. Similarities between this model and nerve have been studied particularly by Lillie (1926) who, on the basis of rather striking analogies, has inferred that nerve and other protoplasmic systems function by reason of a passivating film, of the nature of an oxide film, which covers the cell membrane. When this film is disturbed, particularly when it is electrochemically reduced, the local circuit between the reduced region and adjacent oxidized regions reduces or activates the latter, and in this manner the negative wave is propagated over an initially uniform cell surface without decrement. Refractoriness consists in the reestablishment of this film to a stable condition, due to reactions within the nerve, after which, by a partial reduction, the film becomes less stable, *i.e.* more reactive, during the relatively refractory state, until complete recovery of irritability subvenes.

The mechanism of passivity in metals has not been satisfactorily explained. Evans (1922) concludes that the surface is protected by a layer, perhaps monomolecular, of a metastable oxide not of any recognized composition and possibly of activated oxygen itself. Bennett and Burnham (1917) suppose that an unstable superoxide is adsorbed on the iron surface, and is in this condition relatively stable.

1. Technique.

In the following experiments No. 24 B and S gauge steel spring wire was used in 65 per cent commercial nitric acid except where otherwise specified. The wires

were about 10 cm. long, and were bent at one end which emerged from the acid. They were fitted into the slots of a glass slide-staining dish about 4 × 8 cm. and 3 cm. deep, lying horizontally under the acid, with about 7 cm. of wire immersed. They were customarily stimulated in the tests by touching in the middle with a strip of zinc, the excitation progressing both ways from the point excited. Three wires were used, a polarizing current being sent through the two outside wires, the middle one serving as an indifferent reference electrode (Fig. 1). Readings were made with a milliammeter and a millivoltmeter, which followed the changes rather roughly. To check the precision of the readings, series of potentials were read on a calibrated low sensitivity galvanometer through a high resistance, the galvanometer having a 1/4 second period. Readings were also made on a Leeds and Northrup Type K potentiometer, by tapping the key during the active phase, the balancing potentiometer potential being approached by successive trials. Since the wires remained active for about a second, and since the whole of the

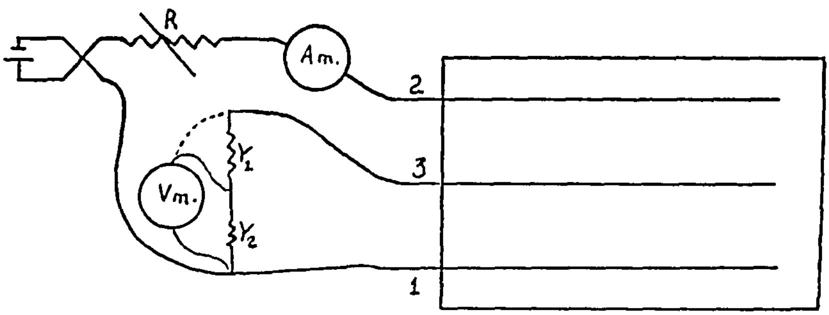

FIG. 1. Arrangement of steel wires in nitric acid for recording effects of polarizing currents upon activation (see text).

short wire used was active within 1/10 second after the middle point had been stimulated, the instruments gave fair approximation to the actual potentials. As a final check upon finer details of potential change during the reaction, photographs of the potential record were taken on the cathode ray oscillograph.

2. *The Active Process in a Short Length of Wire.*

While the active process in this model has been described before (Lillie, 1920), certain details observable in the small model here used seem to be worthy of notice. In the first place, an electrical circuit between active and passive regions is by no means necessary to recovery, for the whole length of wire may be active at one time, and passivate spontaneously. On the other hand, when unstimulated, the wire is not entirely passive. When connected to the cathode ray oscillograph through an amplifier, the apparently passive wire causes a continual flicker in the record, as if small areas were becoming active here and there but were promptly passivated without propagation. After stimulation of both wires (*i.e.* the test wire and the "indifferent" lead) the system is perfectly quiet until

refractoriness is recovered from. These flickers are of too short duration to be recorded on a voltmeter.

When the wire is irritable, and thinly coated with a reddish brown film, stimulation causes an immediate deposit of dark brown or black oxide, and this gets slightly darker during the second that the wire has a negative potential. With the very first of the reaction a few fine bubbles are seen to rise from the wire, and no further gas escapes until the wire repassivates. This is in contrast to the initial passivation, when a fresh wire foams violently during the active phase. Just upon repassivation, a large flock of fairly large bubbles escapes from the wire. At the anode under a polarizing current many more bubbles rise at the initiation of the negative potential, while none rise at the cathode. Why they escape at just these times is not clear, but the phenomenon may be correlated with that at a platinum electrode polarized by a current to the point where gas is about to escape in bubbles. A sudden break of the circuit will cause the bubbles to be released. Possibly a charge on the gas bubbles helps to hold them at the polarized surface, or repels them when the potential changes.

This escape of gas is a constant concomitant of the passivation. If a wire is stimulated while partially refractory, the brown oxide coat spreads only a short distance along the wire, with no production of gas at its initiation. The second crop of bubbles then starts at the now stationary border between active and passive regions, and passivation sweeps over the wire in the reverse direction to the activation wave.

The activated wire becomes negative to the indifferent or passive wire by about 0.83 volt, falls rapidly to about 0.7 volt and falls more slowly from that value until sudden passivation. It then becomes positive by 0.1 to 0.2 volt and recedes to the initial potential, at first rapidly, then slowly. It is completely refractory until this overshoot has nearly disappeared. These potentials will doubtless vary with acid concentration, etc.; Lillie reports lower values for the positive potential of passivation.

3. Polarization Curves of Anode and Cathode.

Of two similar wires polarized by a current, the potential drop from solution to cathode is always less than from anode to solution. The nitric acid apparently depolarizes the cathode by oxidizing whatever products of reduction appear there. In Fig. 2 is shown the curve of polarization of anode and cathode plotted against current. At the voltage corresponding to the abrupt change of curvature of the anode curve, bubbles escape from the anode.

4. Form of the Action Potential under Polarization.

String galvanometer records of the steel wire action potential have been presented by Deriaud and Monnier (1924). These records were

led off from two points along the electrolyte surrounding the wire, as the active process passed. Owing to the fact that local circuits between wire and electrolyte flow far in advance and in the rear of the excited area, these records do not give exact information as to the form of the potential at any one point on the activated wire. As they state, the form of the rise and fall is in their curve logarithmic; but this logarithmicity is largely a function of the changing distance

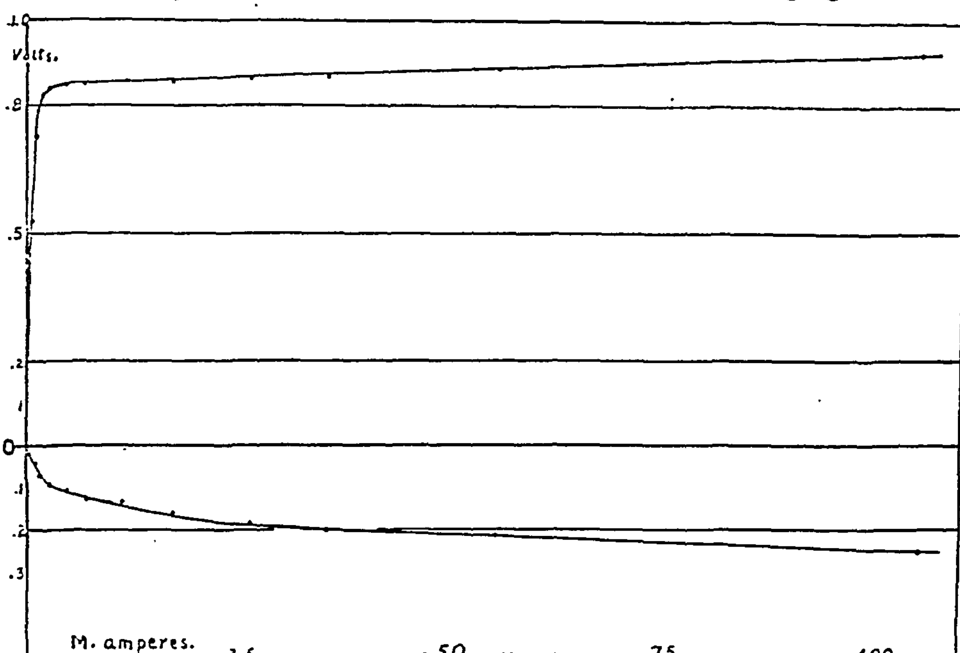

FIG. 2. Polarization curves against an indifferent passive wire (3 on Fig. 1) for anode and cathode. The resistance drop through the bath is ignored, and is relatively small. Potentials recorded at approximate equilibrium for a given applied P.D.

from the active area to the lead, and not alone a function of the activation itself. A tracing of their record is reproduced in Fig. 3, *A*, for comparison with the record from a short length of wire. Records were taken on the cathode ray oscillograph by leading from one passive wire to the ground of the amplifier, from the other to the grid through a high resistance. That is, in Fig. 1, Wire 1 was grounded, and the amplifier connected in place of *Vm.*, the resistance *r* being adjusted appropriately. The time movement of the oscillograph spot was

obtained by charging a condenser in parallel with the vertical oscillograph plates through a two element vacuum tube working at current saturation, the speed being controlled by the filament current of the tube. The movement of the beam of electrons of the oscillograph along the x axis is then practically linear, and can be made as slow as desired. A photographic film was held against the face of the oscillograph by a rubber dam membrane stretched over a ring; the

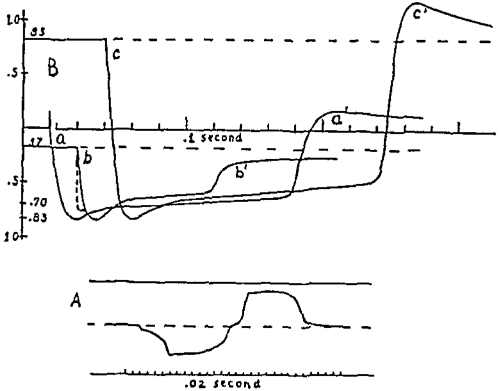

FIG. 3. *A*. Tracing of string galvanometer "diphasic" record from Deriaud and Monnier taken with an unpolarized wire, the leads being from the adjacent solution.

B. Diagram of monophasic potential changes upon activation of the unpolarized wire, (*a*), cathodally polarized to 0.17 volt, (*b*), anodally polarized to 0.83 volt, (*c*), reconstructed from cathode ray oscillograph records and galvanometer readings of potentials. The indifferent lead was another passive wire. Dotted curve on rise of *b* is record of effect of contact of zinc as stimulus to activation.

short circuit of the condenser was broken by opening a key with one hand, and with a strip of zinc held in the other the middle of the wire was stimulated. The electron beam in its course across the tube was then deflected upwards by the amplified fraction of the potential between the activated and inactivated wires.

In our condenser-coupled amplifier only potentials of short duration are virtually undistorted, since the condensers discharge to a

new level under a constant input potential. Control curves could, however, be taken, and subtracted from the records. Such reconstructed potential curves are drawn in Fig. 3, the actual values of the potentials being those obtained by direct measurement with voltmeter and galvanometer. The wires were then polarized to about 1 volt potential difference, and records from both anode and cathode against an indifferent passive wire between them were made as above.

On the rising phase of potential (Fig. 3, *B*) the first irregularity is due to breaking of contact between zinc and iron. The time of this event naturally varies, but can often be gotten into the rising phase itself (dotted line on Fig. 3, *B*, *b*). In such a case, the further rise is due to the activity of the iron itself, and the curve of rise has been drawn as if the stimulation irregularity were absent. The initial rise is accomplished in not over 1/10 second, and part of this time is undoubtedly used in propagation from the point of stimulation to the rest of the wire. The rise of potential at any one point occupies probably not over one half that time. The first maximum of the curve is as near as can be measured at the same potential as that of soft dissolving iron wire against a passive steel wire in the same bath, namely −0.83 volt. The wire then promptly falls to about −0.7 volt, then falls slightly until the very abrupt fall at passivation. The record of the fall is prolonged for the same reason as that of the rise, the recovery sweeping over the wire usually from one end, occupying an appreciable time in transit. The potential overshoots upon passivation by +0.15 to +0.2 volts, its return occupying many seconds (Fig. 3, *B*, *a′*).

In the cathodally polarized model (Fig. 3, *b*) whose potential with respect to the indifferent wire is initially −0.15 volt the potential goes not 0.8 more negative, but about 0.65 negative, that is, it reaches the same final potential, but from a lower initial level. In the anodally polarized wire (Fig. 3, *c*) the potential passes from the initial +0.85 volt, 1.65 volts negative, again arriving at approximately the same potential as the normal, that of active iron. The cathodal curve, though lower, is very markedly shorter in duration, the anodal longer, than the normal. The falling phases, however, show the most striking differences. The cathodal curve fails to return to the potential of the indifferent passive wire, or even to its own initial polarized

potential, but approaches a value 0.1 to 0.15 volt negative to this. The anodal curve, however, overshoots its initial potential as the normal curve does, and returns toward its initial potential from the positive side considerably more rapidly than the normal. These return curves are associated with striking differences in the recovery of these wires from the refractory state.

5. *Refractory State under Polarization.*

A wire 7 cm. long in 65 per cent nitric acid recovers its irritability sufficiently to conduct its whole length in a minute or so. When two such wires are polarized to a potential difference of 1 volt, the cathodal wire remains irritable indefinitely, until stimulated. It then repassivates promptly. Thereafter, however, it remains refractory to ordinary stimuli as long as the polarizing current flows. Its oxide layer is reduced until light yellow in color, but does not disappear. Such reduction does not abolish its initial irritability, in fact, it becomes easier to stimulate.

At the anode, on the contrary, recovery from refractoriness is a matter of seconds. As the current flow continues, the black oxide layer becomes heavier, but the wire can be repeatedly activated at very frequent intervals. Finally, however, with a still stronger polarization (1.5 to 2 volts) the anode can no longer respond. If, now, the circuit is broken, irritability may not be recovered for some hours. By making the wire cathodal, however, it becomes again irritable. If the polarization is less severe the wire will recover its irritability without depolarization from an external circuit, the acid gradually dissolving off the oxide layer.

A severely polarized wire (anodal) may be depolarized in spots by prolonged contact with zinc. These localities then become irritable, but conduction stops at their borders. The potential of any overpolarized wire reaches the level of the normal control wire without becoming irritable.

These refractory phase phenomena are so closely correlated with the potential changes following repassivation as to suggest a causal relationship (Lillie, 1920). It might appear that an initial pronounced positivity of 0.1 to 0.2 volt, followed by a return of the same amount, is necessary for the attainment of irritability (*i.e.* at this

concentration of acid, etc.), but there are objections to this view. This phenomenon does not appear to correspond to the initial deposit, and subsequent partial reduction of an oxide layer, for it takes place at the anode where reducing conditions are not present, and it fails to take place at the cathode, though an oxide layer is formed there in spite of a reducing condition that should, on the above theory, make it responsive.

6. Passivation of Soft Iron at Anode.

Though soft or wrought iron shows no passivity phenomena in acid of 65 per cent or weaker, something resembling this can be produced by anodal polarization. Wrought iron wire as usually found is heavily coated with a smooth hard oxide film, and though such a wire resists the action of nitric acid, its resistance is due definitely to a protective coating of the oxide, and not to passivation strictly speaking. If this coating is removed, the wire remains bright in acid until dissolved, the active evolution of bubbles probably washing away the oxide. When anodally polarized, such a bright wire first reacts violently, then more slowly as an adherent oxide coat forms, and gradually ceases to react. This process is probably not only a true passivation, but also involves mechanical protection by the oxide layer, deposited when the polarization potential is sufficient to prevent the reduction of NO_3 ion, and thus the active evolution of gas. This oxide-coated wire is now irritable, and acts like the passive steel wire, except that its refractory phase is much shorter. After a relatively few stimulations, some spot on the surface of the iron becomes exposed and enters into continuous activity. This reaction now serves as a stimulus, and rhythmic waves of activity occur spontaneously until the oxide coat is dissolved off, whereupon the whole wire dissolves rapidly without passivation. During this rhythmic period, the refractory phase is obviously very short, and each process propagates throughout the wire.

If the acid is made slightly stronger, soft iron may be passivated quite satisfactorily by anodal polarization. At a concentration of 70 per cent acid, a potential of 4 volts through a resistance of 30 ohms passivated a 7 cm. soft iron wire at 70 milliamperes. The wire when first dipped into the acid acquires a brown oxide coat, with evolution of

gas; upon passivation the oxide layer disappears suddenly and the wire becomes bright, accompanied by a sudden copious evolution of bubbles. During the current flow, stimulation causes the typical oxide formation upon activation, with gas evolution upon passivation, the wire becoming bright at each passivation, in contrast to the steel wire which remains coated with oxide. After several stimulations in rapid succession the wire fails to repassivate for some time, though this time may be shortened by stirring. If the zinc is held in contact with the iron, the system becomes rhythmic, and the refractory period is very brief. Often the point at which the wire emerges from the acid fails to repassivate, and rhythmic waves then spread over the wire from this point.

Now if the circuit is opened after thorough passivation of the wire, it remains passive in acid that failed to passivate it without the current, and can be stimulated several times with complete repassivation, the refractory phase being shorter than for the corresponding steel wire. Finally the wire becomes continuously active.

These differences between soft iron and steel are functions of the differences in the *reactivity*, that is, functions of the strength of the acid and of the tendency of the particular metal complex to oxidize in the presence of the nitrate ion. If now a metal which does not react visibly in 65 per cent acid is polarized negatively, one might expect it to become reactive. This was tried with chromel wire, which is a nickel-chromium alloy steel. Chromium steels in general of sufficiently high chromium content have the property of non-corrodibility in nitric acid. When made either anode or cathode, this steel corrodes, becoming coated with a grey-black oxide. Under no conditions could this wire be made irritable in 65 per cent nitric acid. The chromium dissolved in the metallic ferrite of the steel presumably prevents attack of the iron by the acid (Monypenny, 1926). The relative non-reactivity of this system is therefore probably due to the low potential of the reaction between acid and iron, which is insufficient to reduce adjacent regions of the wire, since in the other wires, the negative potential during the excitation is characteristic of iron in a reactive state.

Placed in 20 per cent nitric acid, chromel wire seems to be irritable. The wire remains perfectly bright, but when touched with zinc, a

slight change flashes very rapidly over the wire, with no appreciable production of gas.

7. The Equilibrium between Inactive Metal Electrodes and Nitric Acid.

If a passivated steel wire is raised out of the acid, held in air a few seconds and replaced in the acid, the wire is immediately 0.02 to 0.10 volt positive to a similar undisturbed wire. It slowly becomes more negative, until it reaches the potential of the undisturbed wire. Chromel and platinum wire likewise become more positive when reimmersed after exposure to air. This phenomenon is therefore not a function of the oxidation of the iron, but is rather due to a change at the surface of the metal which takes some time for recovery in acid to the steady state. A probable explanation of this phenomenon is that upon exposure to air, nitrous oxide is oxidized to nitric, or else nitric oxide evaporates into the air, leaving oxygen at the electrode. When immersed, the gases in the solution will again come to equilibrium at the electrode surface, giving a potential characteristic of their equilibrium. At this equilibrium point, either NO_2 or NO might produce a more negative potential than O_2 alone, while O_2 will be reduced to NO_2 by the NO of the solution.

A clean platinum wire immersed in 65 per cent nitric acid is initially 0.18 volt positive to a passivated steel wire, and with time, approaches the potential of the steel. A clean chromel steel wire is upon immersion strongly negative, but becomes positive so rapidly that it is difficult to measure the potential. It reaches a maximum of about 0.12 volt, then slowly returns to the potential of the passive wire. The latter therefore apparently acts, like the less reactive metals, platinum and chromel steel, as an *inert electrode*, and measures a potential; not characteristic of iron, but characteristic rather of the equilibrium between the oxidizing and reducing constituents of the solution.

DISCUSSION.

Laying aside for the time the causation of passivity in metals, the foregoing experiments may be interpreted as if this passivation-activation were a fluctuation between two states of the metal which correspond to a displacement in the electromotive series. Active iron behaves as if it were above hydrogen, *i.e.* more electropositive,

and passive iron as if more noble. The curves show that the change from one to the other is very abrupt, if not immediate.

In nitric acid, metals above hydrogen tend to displace the hydrogen of the acid, forming metal salts, which latter may then reduce more nitric acid forming oxides. Metals below hydrogen, if they react, tend to reduce the acid with direct formation of oxides (Remsen, 1898), which then react with more acid to form metal-acid salts; in either case the final equilibrium arrived at depends upon the physico-chemical state of the system. Iron in the reactive state replaces hydrogen; soft iron stays bright under attack, and while the gas evolved is not hydrogen, it may be looked upon as the reduction product of nitric acid reacting with the hydrogen displaced from the acid. Iron in the passive state, if it reacts at all, apparently forms an oxide directly, or more readily. Passivation is induced in the iron-nitric acid system by anodal polarization, by addition of ferric salts to the acid (Monypenny, 1926), and by increasing the concentration of the acid. All these should be expected to favor oxide formation as compared to simple metal-acid salt formation, either by increase in the concentration of the ferric ion, or by increase in the oxidizing power of the acid, which in high concentration tends even to be unstable. It seems possible that the converse of the electromotive relationship of active and passive iron may hold; that factors which tend to induce oxide formation at the expense of metal-nitrate formation thereby tend to passivate the metal.

It is apparent that anodal polarization, both by concentration of the anion of the acid, and by increasing locally the concentration of the reaction products, that is of metal-acid salt, would tend to depress the formation of this salt, and at the same time to induce the alternate formation of relatively insoluble oxide. However, if the metal were very reactive, the reaction might use up the acid in the immediate vicinity of the wire, effectively reducing the concentration. When once locally activated, the active-passive couple, as Lillie, 1920, has pointed out, tends to reduce the passive region, while it oxidizes the active region. In the soft iron wire in 70 per cent nitric acid activation under the influence of this couple is the only event during which visible oxide formation is observed. If the acid is made weaker, no oxide is deposited on the wire, though it is formed in the reaction.

The active-passive couple therefore may act as an anodal polarization which steps up the potential sufficiently to induce oxide formation, provided the concentration of acid is sufficient, when otherwise the iron would dissolve continuously as the metal acid salt. While the visible oxide so formed is not the passivating oxide film assumed to be responsible for passivation in general, the same conditions might apply to the production of the latter. The *initial* formation of this brown oxide coat seems in general to be necessary to passivation of iron in dilute nitric acid.

The inference that the partial reduction of this passivating film is responsible for the ability of the system to conduct the excitation, while in agreement with the potential changes which accompany irritability changes after response, does not seem reconcilable with the effects of polarization upon the refractory phase. Under the anode, with violent evolution of an oxidized gas and constant deposition of metal oxide, the refractory phase may be so extremely brief as to leave no doubt that an oxidizing rather than a reducing process has shortened the return of excitability. Furthermore, the analogy between the iron wire and nerve, where anodal polarization again *shortens* the absolutely refractory phase, while it prolongs the duration of action potential (Bishop and Erlanger, 1926), leads to the inference that oxidation rather than reduction is the factor which induces the irritable state. The overshooting of the potential after excitation has not been observed at all in nerve, where it should be in evidence if it occurs as a diphasic modification of the monophasic unpolarized wave.

Perhaps a compromise hypothesis can be devised by assuming the unstable oxide adsorbed on the metal surface, as Bennett and Burnham discuss, a process which may take appreciable time, and assuming that the elevated potential just after excitation is due to a temporary imbalance between the constituents of the solution at the oxide surface acting as an electrode. The relatively refractory state might then be due to a *deficiency* of the *labile* oxide, though a more stable and visible oxide may have been formed in the period of activity. This latter oxide probably serves mechanically as a protective coating against rapid attack by acid, and secondarily, is too inert chemically to dissolve readily under the local current of activity.

According to this hypothesis, the cycle of activation may be described as follows: Upon immersion, rapid evolution of gas tends to prevent adherence of an obstructing oxide coat. When either through an adherent oxide coat or through a high concentration of acid, or by reason of a polarizing current, conditions are such that some particularly labile higher oxide can be formed and adsorbed on the iron, this oxide protects the surface, not only mechanically, but electrochemically, acting as an electrode surface which has but slight tendency to an exchange of electrons with the solution. Due however to the unstable character of this oxide, the system is "irritable" to factors which can cause its breakdown, such as the local active-passive couple. The change in the potential during the relatively refractory state may be due to the gradual production of an equilibrium between the labile oxide coat acting as an electrode, and the constituents of the solution.

In the case of the soft iron wire in 70 per cent acid it will be recalled that brown oxide was formed during the negative or active phase in spite of the reducing local circuit, the metal becoming bright again upon passivation. When first immersed, this brown oxide is not adherent, and the metal does not passivate. Acceleration of oxidation by anodal polarization led to passivation, which then returned after activation. It may be inferred that the oxide coat, adherent but doubtless porous, permits of the formation of the passivating substance without the active gas formation which rapid oxidation involves, and this substance can thus be deposited as a protective coating in the interstices of the first coat, and all over the surface as this brown oxide is dissolved away in the acid. Obstruction by one coating might thus be the necessary preliminary to the laying down of the other. On the other hand, upon local activation, as the local current reduces the labile oxide, *gradually* laying bare the metal surface, this surface can be oxidized less violently than when initially immersed, and the first brown oxide coat is thus allowed to adhere instead of being washed away by gas evolution and consequent convection in the fluid.

Activation and passivation may thus involve alternate protective coatings of different properties, the one labile and potentially reactive, passivating the metal electrochemically until disturbed, the other

stable and slightly soluble, protecting the surface mechanically until true passivation can take place. During the first stages after passivation, the labile film *thickens*, and comes to equilibrium gradually with the solution as an inert electrode, while the more stable film dissolves away. The potential of the first hump on the potential wave shown both in Deriaud and Monnier's curves and in our own is presumably that of active iron (0.83 volt); following this the lower potential (0.7 volt) is that recorded from iron still active, but mechanically protected by a coating of brown oxide.

Finally, it is only necessary to mention the correspondence between activity in this system and in nerve, a correspondence repeatedly pointed out by Lillie. The data of this paper show this correspondence to be even more particular. Avoiding the implication that there is any correspondence in the specific chemical reactions involved, the correspondence shown in the general character of the reactivity of the two systems may be extended to the following points.

1. The counter E.M.F. of both systems under a given polarizing current is greater at the anode than at the cathode.

2. Anodal polarization prolongs the duration of activity, cathodal polarization shortens it.

3. Anodal polarization increases the action potential, cathodal polarization decreases it.

4. Anodal polarization shortens the refractory phase; cathodal polarization lengthens it.

5. The effects of *strong* anodal polarization are more severe, in that recovery of irritability is slower, than the effects of cathodal polarization.

6. Cathodal polarization first renders both systems more irritable, with stronger currents non-irritable, to a second stimulus.

One striking difference lies in the forms of the potential waves in the two systems. That of the wire model is noticeably rectangular, that of the nerve has no discontinuity of curvature. It is quite possible, however, that, owing to the reactance involved in the polarizable membranes of nerve, the recorded action potential is not of the same form as the potential actually produced by the nerve mechanism.

SUMMARY.

The active process in a short length of steel wire passivated by 65 per cent nitric acid has been observed under the influence of a polarizing current, and the form of the potential recorded by the cathode ray oscillograph. In the passive wire, 80 per cent of the total potential drop takes place at the anode, 20 per cent at the cathode. The change from active to passive states, as measured by the potential change, is very abrupt compared to the duration of activity and the potential curve at a point on the wire is probably almost rectangular.

The duration of the refractory state is decreased at the anode and increased at the cathode, as in nerve. This fact is against the idea that reactivity after passivation results from a partial reduction of an oxide layer.

Soft iron wire passivated by anodal polarization repassivates after activation in acid of a dilution that fails to passivate it initially. It soon becomes rhythmic with a very short refractory phase, and then reacts continuously. Such a wire exhibits a very sharp alternation between a dark brown oxide coat during activity, and a bright clean surface during passivation.

A passive steel wire in nitric acid shows many of the characteristics of an inert electrode such as platinum, and it may be inferred that, superposed upon the primary passivation potential, there exists an electrode or oxidation-reduction potential equilibrium between the effects of the various constituents of the solution.

It is suggested that the phenomena of nerve-like reactivity in this system may involve an alternation between two protective coatings of the steel wire. During activity, the surface becomes mechanically coated with a brown oxide. If this coating does not adhere, due to gas convection or to rapid solution of the oxide, passivation does not result. Under sufficiently intense oxidizing conditions, a second oxide coat may form in the interstices of the first, and cover the surface as the first coating dissolves off. This furnishes the electrochemical protection of passivation, which is followed by the gradual attainment of electrode equilibrium with the solution.

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THE RESPIRATORY QUOTIENT OF FROG NERVE DURING STIMULATION.

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INTRODUCTION.

Some experiments have recently been described (Fenn, 1927) which successfully demonstrated an increased oxygen consumption of the nerves of dogfish and frogs during stimulation, thus confirming Tashiro (1913) and Parker (1924–25) who measured CO_2 output as well as Downing, Gerard, and Hill (1926) and Gerard (1927, *a* and *b*) who measured heat production. The apparatus, a differential volumeter, consists of two small flasks attached through suitable cocks to opposite ends of a horizontal capillary tube containing a tiny kerosene index drop. If sodium hydroxide is contained in one of these bottles with the nerve, then the index drop moves toward the nerve bottle and measures by its rate of movement the rate of oxygen consumption. The other bottle is empty and serves for temperature compensation. If sodium hydroxide is omitted from the nerve bottle CO_2 is not absorbed and the movements of the drop then indicate the difference between O_2 consumption and CO_2 output. By alternate measurements on the same nerve with and without NaOH, both O_2 and CO_2 changes can be measured. Experiments of this type on dogfish nerve (Fenn, 1927) yielded values for CO_2 considerably less than those for O_2, giving an apparent R.Q. of 0.7 to 0.8. A rough estimate showed that the true R.Q. might nevertheless be very nearly 1, because of the CO_2 retained as the CO_2 tension inside the respiration chambers progressively increased.

In similar experiments on frog nerves reported in this paper,[1]

[1] The results of these experiments were reported to the American Physiological Society, April, 1927, at Rochester, N. Y., *Am. J. Physiol.*, 1927, lxxxi, 476.

an attempt has been made (*a*) largely to avoid this CO_2 retention by studying the nerve at high CO_2 tensions; and (*b*) to calculate the CO_2 retained, from measurements of the CO_2 dissociation curve of nerve.

Method.

The differential volumeter used for these experiments is a very sensitive one, having a capillary about 0.3 mm. in diameter, the bottles holding only about 3.5 to 4 cc. A movement of 1 cm. in the

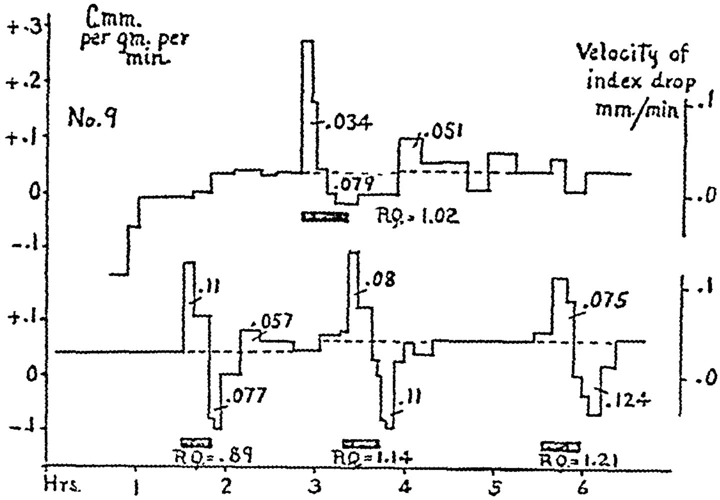

FIG. 1. Rates of oxygen consumption minus rates of carbon dioxide output plotted as ordinates against time. A rise in the curve indicates a relative increase in the oxygen or decrease in the carbon dioxide. Figures on the graph represent volumes in c. mm. per gm. nerve per minute of stimulation corresponding to the areas indicated on the graph (See Table I, Experiment 9, *a*, *b*, *c*, *d*, for calculation of the R.Q.).

capillary corresponds to an O_2 consumption of $2 \times 0.00073 = 0.00146$ cc. The two sciatic nerves from one good sized frog (*R. pipiens*, 3 to $3\frac{1}{2}$ inches long) were usually used. Reference must be made to the previous paper for details of the technique.

Results.

A typical experiment of the type described is plotted in Fig. 1. Ordinates represent rates of movement of the index drop or rates of volume change per gm. of nerve per minute. Abscissæ represent

times; hence areas represent volumes. In this case 80 mg. of nerve were used in an atmosphere of 20 per cent CO_2 in oxygen without NaOH. The bottle contained in addition 0.1 cc. of 0.1 M HCl to absorb any possible ammonia. Under such circumstances the retention of carbon dioxide is small and the oxygen consumed is only slightly greater than the carbon dioxide output, as shown by the very small rate of movement of the index drop. The two nerves were stimulated four times for 20 or 30 minute periods as indicated by the black marks.

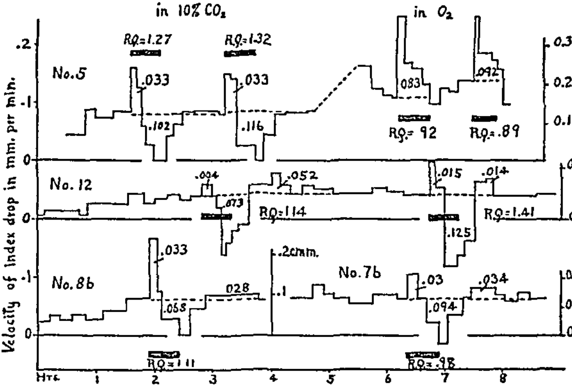

FIG. 2. Ordinates and abscissæ as in Fig. 1. The results of stimulation periods from Experiments 5, 7*b*, 8*b*, and 12 of Table I are plotted. All the nerves were in 6 to 10 per cent CO_2 atmosphere except in the last two stimulation periods of Experiment 5, as noted.

The stimulating current came from the secondary of a Harvard induction coil, coil distance 13 cm., and with a P.D. of 0.56 volts across the primary terminals (weak stimulation). A glance at Fig. 1 shows the characteristic effect of stimulation—first an abrupt rise, then a more prolonged fall outlasting the stimulation period and followed by a slight rise which is scarcely greater than the experimental error and which does not always appear. The area of each of these rises and falls is indicated in Fig. 1 in c. mm. per gm. of nerve per minute of

stimulation.[2] These volumes are all small (varying from 0.03 to 0.12 c. mm.) compared to the excess oxygen consumption, which in my experiments has an average value of 0.32 c. mm. per gm. nerve per minute of stimulation. In the first period of stimulation (Fig. 1) the two rises represented volumes of 0.034 and 0.051 or a total of 0.085 c. mm.; the fall was 0.079, the difference being 0.006 c. mm. If the oxygen be taken as 0.3 c. mm. (from other experiments) then the apparent CO_2 must have been $0.3-0.006 = 0.294$. The other periods of stimulation in Fig. 1 have been calculated in the same way and the values so obtained are inserted in the

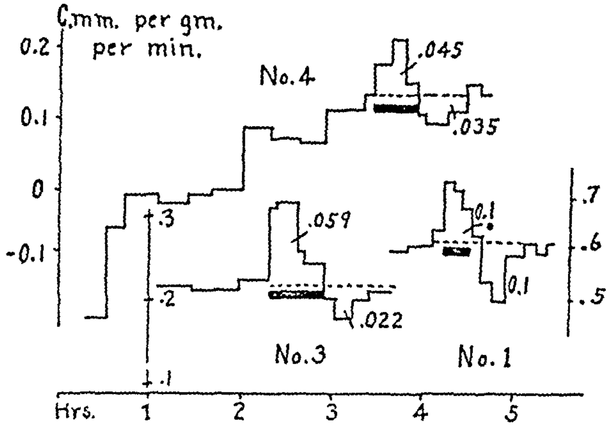

FIG. 3. Results of stimulation periods from Experiment 1, 3, and 4 of Table I. Abscissæ and ordinates as in Figs. 2 and 3. All three nerves were in an atmosphere of oxygen without NaOH for absorption of CO_2. Note that the resting rates are higher than in Figs. 2 and 3 as are also the magnitudes and durations of the initial rises.

figure. The calculation of the true R.Q. will be described in connection with Tables I and II in which the experiment of Fig. 1 is included as No. 9, *a*, *b*, *c*, and *d*.

The results of eight other similar stimulation periods are plotted in Fig. 2. In the upper graph (No. 5 of Table I) the last two stimulation periods were run in oxygen rather than in a CO_2 mixture. The results are similar except that the rise is greater and the fall is less

[2] Strictly, these figures are not areas but rates assumed to persist for the duration of the stimulation period. True areas are obtained by multiplying by the duration of the stimulation in minutes.

and somewhat delayed. In the second graph (Fig. 2) are the results of an experiment in 10 per cent CO_2, in which, in both stimulation periods, the fall was much greater than the rise, indicating an excessive CO_2 output. It would seem possible that in this case recovery was incomplete and some of the CO_2 eliminated was derived from preformed carbonates.

In Fig. 3 are plotted the results of three stimulation periods on three different pairs of nerves kept in an atmosphere of oxygen with only traces of carbon dioxide. These experiments show clearly a distinct fall following stimulation. Similar falls would presumably have appeared with equal clearness in the two stimulation periods in O_2 of Fig. 2, Experiments 5 *c* and *d*, if the readings during recovery had been continued longer. Both in oxygen and in 10 per cent CO_2 there is, therefore, a period in which the CO_2 output exceeds the oxygen consumption. The presence of CO_2 serves merely to increase the magnitude of this CO_2 outburst and to cause its appearance within 10 to 15 minutes of the beginning of stimulation rather than at the close of stimulation as in Fig. 3. The interpretation of these results will be discussed in more detail below.

The upper graph in Fig. 3 illustrates the characteristic initial variations in the resting rate. At first in the absence of NaOH and of CO_2 the *gas in the nerve chamber increases* in volume and the drop moves away from the nerve chamber This might be due to an initial "gush" (Parker, 1924–25) of CO_2 without a similar increase in O_2, but the fact that it may occur also in an atmosphere of 10 per cent CO_2 (Fig. 1 upper graph) suggests that some other factor may be involved. After about 1 hour this movement ceases and a slow movement toward the nerve chamber begins (plotted as a positive rate in Fig. 3). This rate usually increases very gradually throughout the course of an experiment. In the presence of CO_2 (5 to 20 per cent) the resting rate of movement of the index is never greater than 1 mm. in 10 minutes. In pure oxygen, however, it may be as high as 2 or 3 mm. in 10 minutes. This calculates out to a volume change of 0.1 to 0.2 c. mm. per gm. per minute. The high rate of 0.6 c. mm. shown in the third period of Fig. 3 is exceptional. The oxygen consumption of these same nerves, subsequently measured, was also very high, 1.8 c. mm. per gm. per minute. It may have been also an exceptionally well buffered nerve, or its R.Q. may have been unusually low.

The Respiratory Quotient of the Excess Metabolism.

Further consideration of the time relations of the oxygen consumption and carbon dioxide output will be reserved until after the method

TABLE I.

The Respiratory Quotient of the Excess Metabolism of Stimulated Nerve.

1	2	3	4	5	6	7	8	9	10	11	12
Experiment No.	cc. × 10^{-5} per gm. per minute of stimulation			Weight of nerve	CO_2 in gas	Slope	CO_2 retained	cc. × 10^{-5} per gm. per minute of stimulation			R.Q.
	O_2	O_2-CO_2	CO_2					CO_2 retained by nerve	CO_2 retained by HCl	CO_2 corrected	
				mg.	*per cent*		*per cent*				
1	25*	0	25.0	74	0	1.5	23	5.8	—	30.8	1.23
2	50*	−4.2	54.2	59	0	1.5	19	10.3	—	64.5	1.29
3	26.7*	3.7	23.0	72	0	1.5	23	5.3	—	28.3	1.06
4	26*	1.0	25.0	119	0	1.5	38	9.5	—	34.5	1.33
5*a*	30	−6.9	36.9	89	10	0.19	3.6	1.3	—	38.2	1.27
5*b*	30	−8.3	38.3	89	10	0.19	3.6	1.4	—	39.7	1.32
5*c*	30	+8.3	21.7	89	0	1.5	28	6.1	—	27.7	.92
5*d*	30	+9.2	20.8	89	0	1.5	28	5.8	—	26.6	.89
6*a*	32*	−18.3	50.3	57	10	0.19	2.3	1.2	—	51.5	1.61
6*b*	32*	−11.3	43.3	57	10	0.19	2.3	1.0	—	44.3	1.39
7*a*	30	+2.5	37.5	69	10	0.19	2.8	1.1	1.5	40.1	1.34
7*b*	30	+3.0	27.0	69	10	0.19	2.8	0.8	1.5	29.3	.98
8*a*	30	−2.6	32.6	95	10	0.19	3.8	1.2	1.4	35.2	1.17
8*b*	30	−0.7	30.7	95	10	0.19	3.8	1.2	1.4	33.3	1.11
9*a*	30	+0.6	29.4	80	20	0.14	2.3	0.7	.6	30.7	1.02
9*b*	30	+9.0	21.0	80	20	0.14	2.3	0.5	.6	26.6	.89
9*c*	30	−3.0	33.0	80	20	0.14	2.3	0.8	.6	34.4	1.15
9*d*	30	−4.9	34.9	80	20	0.14	2.3	0.8	.6	36.3	1.21
10	30	−1.1	31.1	72	0	1.5	23.0	7.2	1.1	39.4	1.31
11	30	−6.7	36.7	123	10	0.19	5.0	1.8	—	38.5	1.28
12*a*	30	−1.7	31.7	125	6	0.20	5.4	1.7	.7	34.1	1.14
12*b*	30	−9.6	39.6	125	6	0.20	5.4	2.1	.7	42.4	1.41
Average											1.19

of calculation is described. This involves the determination of the respiratory quotient, after correcting for the CO_2 which is retained by the nerve. The experiments used for this purpose are tabulated in Table I. The value used for the excess oxygen consumption due

to stimulation is given in Column 2. Figures marked with an asterisk were experimentally observed. Others are assumed on the basis of many other experiments on similar nerves. Column 3 gives the observed value of $O_2 - CO_2$ or the algebraic sum of all the deviations from the resting base line, such as those illustrated in Figs. 1 to 3. Column 3 gives the difference between the O_2 and the $O_2 - CO_2$ values, representing, therefore, the observed output of CO_2 per gm. nerve per minute of stimulation. As already explained this is not the total CO_2, especially in experiments run in pure oxygen, because CO_2 is retained by the nerve as the CO_2 tension rises in the bottle. The amount retained will depend upon (1) the rate with which the CO_2 tension rises and (2) the slope of the CO_2 dissociation curve of nerve. If A is the cc. CO_2 liberated (*i.e.*, observed) per gm. per minute by a nerve of weight, w gm., in a bottle of 3.6 cc., then the rate of increase of the CO_2 tension is $wA/3.6 \times 760$ or $211\,wA$. The slope of the CO_2 dissociation curve of nerve is taken from measurements to be reported elsewhere. It is expressed in Table I (Column 7) in cc. of CO_2 absorbed by 100 gm. of nerve for 1 mm. Hg rise in CO_2 tension when the nerve is in an atmosphere containing CO_2 at the concentration shown in Column 6. Examination of Columns 6 and 7 will show that the slope is high (1.5 cc.) in pure oxygen but decreases rapidly to 0.2 cc. at 6 per cent CO_2 and to 0.14 cc. at 20 per cent. Above 6 per cent therefore it is not very different from the slope, similarly expressed, for pure water at 22°C., which is 0.109 cc. The CO_2 retained by 100 gm. of nerve per minute in the bottle used when A cc. is liberated per minute, will therefore be $211\ A\ w \times$ slope. The results of this calculation are given in Column 8. Thus in the first example of Table I it is found that, with a nerve of 74 mg., when 25.1×10^{-5} cc. of CO_2 is observed to accumulate in the bottle, another amount of CO_2 equivalent to 23 per cent of this value must have accumulated inside the nerve due to the buffering of the tissues. The CO_2 retained per gm. of nerve per minute of stimulation is given in Column 9, this being the product of Columns 8 and 4. Column 10 gives the rate with which CO_2 would be expected to dissolve in the small amount of HCl which was contained in the respiration chamber in Experiments 7, 8, 9, 10, and 12 in order to absorb any possible ammonia. It is calculated from the solubility of CO_2 in water and

from the rate with which the CO_2 tension would be increasing in the bottle due to the excess metabolism resulting from stimulation (Column 4).[3] Column 11 gives the corrected CO_2 (Columns 4 plus 9 plus 10). Column 12 gives the respiratory quotient calculated from Columns 2 and 11.

The average value for the R.Q. from Table I is 1.19. The significance of this figure may now be considered. The method is open to a number of objections.

1. There is considerable error in calculating the CO_2 retained, particularly in pure oxygen, where the slope of the dissociation curve is high but is rapidly decreasing during the experiment as the CO_2 tension rises. Moreover, by a calculation previously outlined (Fenn, 1926–27) it can be shown that the CO_2 tension at the inside of these nerves may be 3 to 4 mm. Hg. higher than at the periphery. The actual slope which should be used for correction therefore varies from 0.8 to 2.3 cc. at different times after the beginning of the experiment, and at different parts of the nerve. (The tension of CO_2 increases in the respirometer at an approximate rate of 1 mm. Hg. per hour.) Nevertheless, using the average value of 1.5 for the slope, the calculated R.Q.'s for the experiments in oxygen do not seem to differ significantly from those run in an atmosphere of carbon dioxide where the corrections are almost negligible. Even if these corrections are neglected one still finds some values for the R.Q. which are greater than 1.

2. Except in a few cases, shown by an asterisk in Table I, Column 2, the values for the oxygen consumption were not directly measured but were assumed from other experiments with similar nerves. This became necessary because so much time was required to get a good base line before stimulation and to get a complete recovery after stimulation. Moreover the oxygen consumption observed at zero CO_2 tension is assumed valid for the experiments at high CO_2 tensions where a lower value might be expected (Hyman, 1925, for example). Since, however, it is the difference between oxygen and carbon dioxide

[3] If the actual volume of HCl used was 0.1 cc. and the weight of the nerve was 0.07, then the volume of HCl was taken to be $\frac{0.1}{0.07}$. The rate of solution in HCl is therefore expressed also in cc. *per gm. of nerve* per minute.

which is directly measured, a considerable error in the absolute values of O_2 makes little difference compared to other errors involved and could in any case change only the magnitude, not the direction of the deviation of the observed R.Q. from 1.0.

3. A very small error in determining the resting rate or base line may make a very large error in the value obtained for $O_2 - CO_2$. This is due to the fact that the volume changes which must be determined are spread out over considerable time. If the base line is located a little too high it may diminish somewhat the magnitude of the observed rise and simultaneously increase markedly the magnitude of the observed fall. Study of the graphs of Figs. 1 to 3 in this paper will show many cases where the base line seems very arbitrarily chosen. No. 8*b* (lower left hand graph of Fig. 2) is a good example. If the base line had been taken slightly higher the secondary rise of 0.028 c. mm. would have completely disappeared and $O_2 - CO_2$ would have been perhaps 0.07 instead of 0.007 and the R.Q. would have been 1.3 instead of 1.09. It must be admitted that the prolonged recovery is very unfortunate for the method of analysis used. On the other hand these errors must tend to average out and there are certain experiments (*cf*. Fig. 2, No. 12) in which no possible change in the base line could make the R.Q. = 1 or less.

4. A fourth objection is the most difficult to evaluate. The method is based upon the assumption that when the rate of movement of the index drop returns permanently to normal the recovery is complete and the nerve is in exactly the same essential condition as before stimulation. This assumption is made particularly doubtful by the high values averaging 1.19 which were obtained for the R.Q. of the excess metabolism. One explanation which suggests itself for this value is an accumulation of acid within the nerve which breaks up preformed carbonates. To measure directly this change in preformed carbonates, however, as in Warburg's method, one must measure a small difference in a relatively large value[4] and one must use several samples of tissue which are assumed identical. Moreover without

[4] The total CO_2 liberated from 1 gm. of nerve by immersion in acid is about 0.1 cc. The extra CO_2 ($O_2 - CO_2$) liberated in Experiment 12*b*, Table I, was $9.6 \times 30 \times 10^{-5}$ or 0.0029 cc. per gm. of nerve, which is less than the error in determining the total CO_2.

many samples one cannot get the rate of CO_2 output from moment to moment. Attempts to establish an increased acidity in nerve by changes in its CO_2 dissociation curve have not proved quite accurate enough for the purpose and have given conflicting results.

Other possible explanations for this high R.Q. are: (1) oxidation at the expense of some reducable substance other than O_2 (*cf.* glutathione) which is not completely oxidized again in recovery; (2) other

TABLE II.

The Respiratory Quotient of Resting Nerve.

Experiment No.	cc. $\times 10^{-5}$ per gm. per minute			CO_2 retained	cc. $\times 10^{-5}$ per gm. per minute			R.Q.
	O_2	O_2-CO_2	CO_2	*per cent*	CO_2 retained	CO_2 in HCl	CO_2 corrected	
1	170*	60	110	23	25	—	135	(.79)
2	235*	65	170	19	32	—	202	(.86)
3	83*	22	61	23	14	—	75	(.90)
4	64*	12	52	38	20	—	72	(1.13)
5a	130	12	118	3.6	4	—	122	.94
5c	130	19	111	28	31	—	142	(1.09)
6	106*	26	80	2.3	1.8	—	82	.77
7	130	7	123	2.8	3.4	5.7	132	1.02
8	130	8	122	3.8	4.6	5.6	132	1.02
9	130	5	125	2.3	2.9	2.9	131	1.01
10	130	15	115	23.0	26.0	4.0	145	(1.12)
11	130	14	116	5.0	5.8	—	122	.94
12	130	4	126	5.4	6.8	2.9	136	1.05
Average............								0.97

unreversed transformations yielding O_2, such as a transformation of carbohydrate to fat; or (3) a change in the nature of the resting metabolism. A resting R.Q. of $\frac{1.24}{1.30} = 0.95$ which during stimulation changes to an R.Q. of the *total* metabolism of $\frac{1.24 + .36}{1.30 + .30} =$ 1.0 would give an excess R.Q. of $\frac{0.36}{0.30}$ or 1.2. The accuracy of my determinations of the resting R.Q. as equal to 0.97 is not quite sufficient to preclude this plausible possibility.

The Respiratory Quotient of Resting Nerve.

In Table II an analysis is made of the observed values for the resting $O_2 - CO_2$ rate from the same twelve experiments included in Table I. The method of calculation of the CO_2 correction is the same as before. The average of all the values for the respiratory quotient is 0.97. Here again the difficulties involved in determining the slope of the CO_2 dissociation curve in experiments in oxygen makes these results particularly unreliable and they are accordingly enclosed in brackets. The average of the results for the other seven experiments gives a value of 0.96. Omitting No. 6, the results vary only from 0.94 to 1.05. There is no obvious reason for believing that these values do not represent the true respiratory quotient of resting nerve, although it is possible that future experiments may demonstrate a progressive increase in the acidity of nerves kept under the conditions of these experiments and hence point to a liberation of preformed CO_2.[5]

The Time Course of the Gas Exchange of Stimulated Nerve.

While these experiments still leave the question of the true respiratory quotient of the excess metabolism in some doubt they do give a good idea of the time course of the gas exchange. This may be diagrammed as in Fig. 4. Ordinates represent c. mm. per gm. per minute of stimulation, the period of stimulation being 30 minutes long. The two observed curves are those in solid lines for oxygen and for $O_2 - CO_2$. The former shows a gradual rise to a maximum rate of 0.32 mm. and a gradual falling off in recovery. The total area under the curve ($0.32 \times 30 = 9.6$ c. mm.) is the same as if this maximum rate had been maintained for exactly 30 minutes and had then fallen instantly to zero. The $O_2 - CO_2$ curve shows an initial rise (area $= 0.039 \times 30 = 1.17$ c. mm.), a more prolonged fall (area $= 0.112 \times$

[5] Since the completion of these experiments (March, 1927) I have had a personal communication from Dr. Gerard who has been doing similar experiments by a modified method in Meyerhof's laboratory in Berlin. He reports an R.Q. of the excess metabolism of 1.0 and resting values less than this. While his absolute values are lower than mine there is agreement in finding the excess R.Q. higher than the resting.

30 = 3.36 c. mm.), and a small, much delayed, and somewhat doubtful rise (area = 0.04 × 30 = 1.2 c. mm.). The magnitude of these areas are averages taken from all the experimental curves which have been obtained including those in Figs. 1 and 2. The difference between the oxygen and the $O_2 - CO_2$ curves is plotted (solid line) as the CO_2 output in 10 per cent CO_2 mixtures. There is evidently a characteristic delay in the appearance of the CO_2. The true CO_2 curve is slightly higher than this one by 1 to 2 per cent, due to retention of CO_2. Likewise the CO_2 output in oxygen is lower than this curve. One possible curve for CO_2 output in oxygen is plotted in Fig. 4 (broken

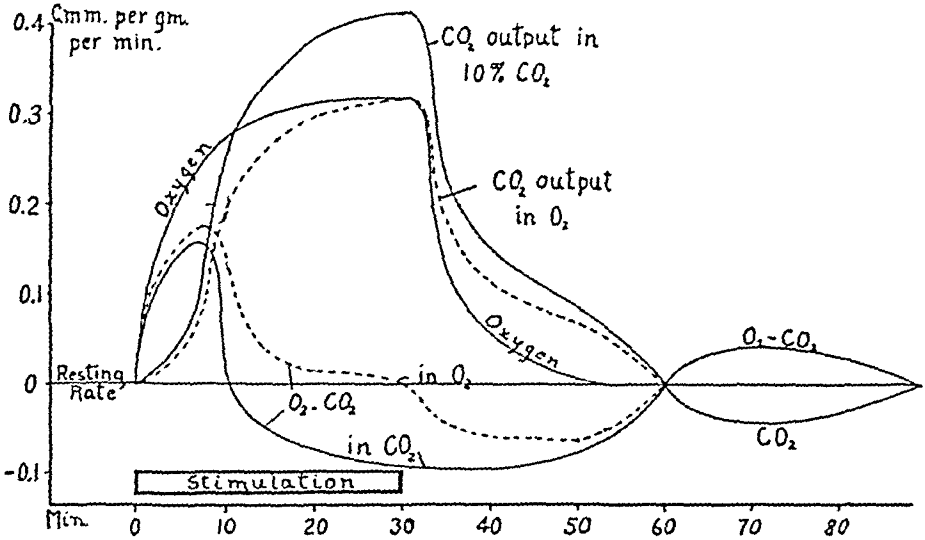

FIG. 4. Diagrammatic summary of graphs of Figs. 1 to 3 (see text).

line) on the reasonable assumption (*cf.* Table I, Column 8, Nos. 1 to 5) that 22 per cent of the CO_2 liberated in an atmosphere of 10 per cent CO_2 would be retained in oxygen. From the difference between this curve and the oxygen curve, another dotted curve is drawn for the $O_2 - CO_2$ in oxygen. The exact *shape* of such a curve depends upon both the size of the nerve and *the size of the bottle,* for this determines the rate of increase of the CO_2 tension. It is obvious, however, that the appearance of negative rates immediately after stimulation agrees well with the experimental curves plotted in Fig. 3 for $O_2 - CO_2$ in oxygen. The areas of the initial rise and the secondary fall in

seven curves (in O_2) similar to those in Fig. 3 were 2.6 and 1.7 c. mm., respectively. The areas under the calculated curve in Fig. 4 are 2.0 and 1.35 c. mm, *i.e.*, slightly higher but of the same relative magnitudes. This diagram would seem therefore to justify the interpretation which has been offered for the experimental curves.

In Fig. 4 there is diagrammed a small rise in the base line beginning a half hour after the close of the stimulation period and lasting for another half hour. This feature of the curves is absent in some, doubtful in others, but present with certainty in a few (*cf.* Fig. 2, No. 12, one of the most reliable of the experiments from this point of view because of the large weight of nerve used). The interpretation of this fact is not clear.

That the peculiar time course of these curves for $O_2 - CO_2$ is readily explained in its chief features at least from considerations of the diffusion coefficients and solubilities of O_2 and CO_2 in nerve tissue, is made evident from the theoretical curves which are plotted in Fig. 5 and which show the same general shape as that found experimentally. The calculation is based upon the fact that although CO_2 diffuses 30 times as fast as oxygen (Krogh, 1918–19),[6] its solubility in the nerve is also 48.5 times as great, *i.e.* for the same change in gas content of the nerve the diffusion gradient is 48.5 times as great for the oxygen as for the CO_2. Hence oxygen would tend to diffuse *in* 48.5/30 or 1.62 times as fast as the CO_2 would diffuse *out*. The adsolute time relations of the diffusion are derived from measurements of the CO_2 dissociation curve of nerve in which it was observed that when CO_2 was suddenly admitted to the nerve chamber, formerly containing only O_2, it diffused in according to a curve which was approximately exponential in form. The diffusion of CO_2 may therefore be represented by the equation, $y = y_0 e^{-\frac{t}{\theta}}$, where the time

[6] The dimensions of Krogh's "diffusion constants" are sq. cm. per unit time *per atmosphere head of pressure*. The usual dimensions of these diffusion coefficients are sq. cm. per unit time. If Krogh's figures are divided by the absorption coefficients of the gases in nerve (cc. gas per cc. nerve per atmosphere pressure) one obtains the true diffusion coefficients which are wanted for this problem. The diffusion coefficient for O_2 is greater than that for CO_2. (See article by Barr, G., Diffusion through membranes, in Glazebrook's Dictionary of physics, v, 135.)

constant, 6, is the average of 11 determinations as follows: 12, 8, 4, 5, 3, 7, 6, 6, 7, 4, 8.[7] The solubility of oxygen in nerve is taken as equal to its solubility in water at 22°C., *i.e.* 0.02988 cc. per atmosphere pressure per cc. of water. The solubility of CO_2 from 10 per cent CO_2 mixtures by actual measurement was 1.45. The time constant for the diffusion in of oxygen should therefore be $6 \times \frac{30}{48.5} = 3.7$.[8]

Now, assuming a certain stretch of nerve in which at time, $t = 0$, a certain volume of oxygen, V_{O_2} is consumed, and a certain volume of CO_2, V_{CO_2}, is formed, then the value of $O_2 - CO_2 = V$ outside the nerve at time, t, will be

$$V = V_{O_2}e^{-\frac{t}{3.7}} - \left(V_{CO_2} - V_{CO_2}e^{-\frac{t}{6}}\right) \tag{1}$$

and the rate of volume change outside will be

$$\frac{dV}{dt} = \frac{V_{CO_2}}{6}e^{-\frac{t}{6}} - \frac{V_{O_2}}{3.7}e^{-\frac{t}{3.7}} \tag{2}$$

This formula, with different time constants, fits the experimental facts for muscle fairly well when the muscle is stimulated for only a few seconds.[9] The nerves, however, are stimulated for 30 minutes. Such a stimulation period may be regarded as a series of thirty 1 minute periods, for each of which the above equation will apply. The graph of this equation has, therefore, been plotted out and the *average* value of the rate for each minute from 0 to 30 has been estimated graphically. Adding together all the individual minute changes, the rate of volume change (or $O_2 - CO_2$ per minute) for the whole stimulation period can be estimated. The curves of Fig. 5 are the result. It is believed that all the experimental curves should lie approximately between these two extremes, the upper one representing

[7] This equation is theoretically incorrect but accurate enough for the present purpose. The experimental agreement with the theoretical equation will be described in a later paper.

[8] Gerard, 1927, *b*, notes a calculation of the rate of saturation of nerves with oxygen which would indicate a time constant much smaller than this, 0.5 instead of 3.7. My CO_2 figure is certainly nearly correct and oxygen could hardly diffuse much faster. Possibly the calculation applied to very small nerves.

[9] Fenn, W. O., 1927–28, *Am. J. Physiol.*, in press.

an R.Q. of 1.0 and the lower an R.Q. of 1.2. For the latter curve one uses the same equation but inserts 120 for V_{CO_2} instead of 100. Using 100 for V_{O_2} the result comes out in per cent of the total excess oxygen consumption.

The experimental diagram of Fig. 4 was drawn before the theoretical interpretation of Fig. 5 was worked out. Aside from the general shape of the curves the agreement in the time relations is noteworthy. It would seem to indicate that diffusion factors were sufficient to

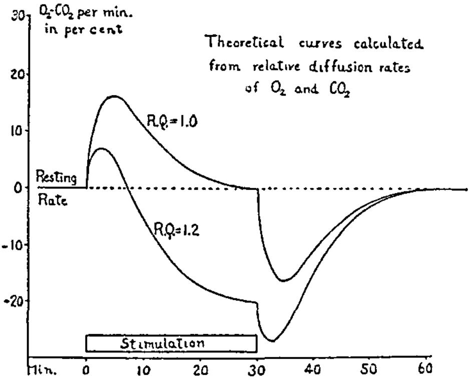

FIG. 5. Theoretical curves suggested in explanation of the experimental curves of Figs. 1 to 3. Ordinates represent changes of $O_2 - CO_2$ per minute expressed in per cent of the total excess oxygen consumption.

account for the recovery processes in nerve after stimulation. A slightly delayed oxygen utilization after stimulation as well as a delayed intake is possible but is not demonstrated in my experiments. There could be no oxygen lack inside the nerves even during stimulation (Fenn, 1926–27).

In the graphs of Fig. 3 particularly there is to be seen a marked fall in the curve immediately after the end of stimulation. This feature is well explained by the theoretical curves of Fig. 5. Too much stress should not be laid, however, upon the exactness with which

Fig. 5 reproduces the experimental curves. There is also one rather striking difference which may be significant. As diagrammed in Fig. 4 the initial rise of the curve for $O_2 - CO_2$ in 10 per cent CO_2 is nearly 50 per cent of the maximum oxygen rate. In the theoretical curves (Fig. 5) this initial rise is only 6 to 16 per cent of the excess oxygen rate. This might be due to a delay in the formation of CO_2 after the actual consumption of the needed oxygen. It might also be explained by a difference in the diffusion rates for O_2 and CO_2 greater than that assumed.

Evidence has been brought forward by Tashiro (1922) and by Winterstein and Hirschberger (1925) that ammonia is liberated from nerve in increased amounts during stimulation. In order to avoid errors from this source some of the experiments of Tables I and II were run with a small amount of 0.1 M HCl in the nerve chamber, as already mentioned. No certain difference could be observed between these experiments and others in which the HCl was omitted. The conclusion must be drawn that under the conditions of my experiments inappreciable amounts of ammonia are eliminated. There remains the possibility that ammonia might maintain a very low and constant vapor pressure (not increasing on stimulation) in an empty bottle and so distil over into HCl if that solution were present.

SUMMARY.

1. By means of a differential volumeter the increased oxygen consumption and the increased carbon dioxide output of frog nerve during and after stimulation have been observed.

2. Measurements of the R.Q. of nerve by this method are complicated by the retention of carbon dioxide. Attempts were made to avoid this (*a*) by studying the nerves at high CO_2 tensions to make the retention small and (*b*) by calculating the amount of CO_2 retained from the carbon dioxide dissociation curve of nerve and applying this value as a correction.

3. The results of both those methods when averaged together give an R.Q. of the excess metabolism of 1.19 and an R.Q. of the resting nerve of 0.97.

4. Observations on the time course of the gas exchange during

stimulation indicate a delay in the appearance of the extra carbon dioxide output relative to the oxygen intake.

5. Very similar time curves can be calculated from the diffusion coefficients and the solubilities of the oxygen and the carbon dioxide.

I am greatly indebted to Mr. W. B. Latchford for assistance in these experiments.

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Since first correcting this proof, Gerard's paper on oxygen consumption has appeared (*Am. J. Physiol.*, 1927, lxxxii, 381), unfortunately too late for comment.

DISSIMILARITY OF INNER AND OUTER PROTOPLASMIC SURFACES IN VALONIA.

By W. J. V. OSTERHOUT, E. B. DAMON, AND A. G. JACQUES.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Accepted for publication, September 20, 1927.)

The protoplasm of *Valonia macrophysa* forms a delicate layer, only a few microns thick, which contains numerous chloroplasts and nuclei. The outer surface is in contact with the cell wall, the inner with the sap of the large central vacuole. As far as microscopic observation goes, there is no difference between the inner and outer surfaces of the protoplasm, but the measurements of potential differences described in this paper indicate that they are not alike.

These measurements were made in the following manner. A fine glass capillary, filled with cell sap[1] or artificial sap,[2] was inserted in the cell (Fig. 1) which was connected to a Compton electrometer in the manner shown in Fig. 2. The calomel electrodes, A_1 and A_2, were filled with saturated KCl and dipped into the beakers B and E containing saturated KCl solution. They were introduced into the chain simply to protect the solution in C and D from excessive contamination by the saturated solution of KCl. For the same reason the solution in H was always maintained at a higher level than in B, and the solu-

[1] The sap has the following composition, expressed as per cent (molar) of halide (which is about 0.6 M):

Cl + Br	100.00
Na	15.08
K	86.24
Ca	0.288
Mg	Trace ?
SO_4	Trace ?
Organic matter, parts per thousand	1.433

Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 228.

[2] This contained NaCl, KCl and $CaCl_2$ in the same proportions as the natural sap. Its halide content was 0.61 M.

tion in D at a higher level than in E, so that any flow in the strings would be away from C and D. In the chain C acted as a support for the cell. The beaker D contained the sap which was in contact with the outside of the cell. The sap in D was occasionally natural sap, but artificial sap was usually employed since it was more convenient and gave the same results as the natural sap.

The beakers B, D and E were supported on dry inverted beakers and the cell holder C on a block of paraffin. All parts of the measuring apparatus (electrometer, switches, calibrating potentiometer, etc., as well as the chain itself) were placed on metal plates (copper or zinc) which were connected together and grounded, and which acted as a shield. The apparatus and its connections were, of course, carefully insulated from the shield. This is the method recommended by White.[3] Total shielding was not attempted. The calomel electrodes were connected through mercury wells to a Compton electrometer.

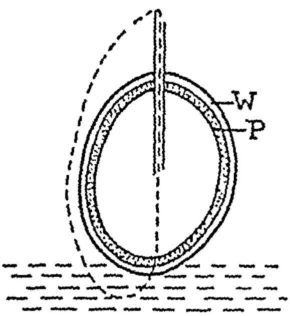

FIG. 1. Diagram of the main circuit in a cell of *Valonia macrophysa* impaled on a capillary and in contact with a solution at its lower surface (W, cell wall; P, protoplasm).

The electrometer was read by means of a lamp and scale, and was calibrated frequently during the work by means of a potentiometer. As the curve obtained by plotting voltage against deflection was not a straight line, the deflections falling off at the higher voltages, calibration was carried out at a voltage somewhere near the potential difference being measured at the time. The sensitivity was between 1.5 and 2 meters per volt. By means of a double pole double throw switch it was possible to have the needle swing in either direction from zero when a potential of the same polarity was applied. This was useful in eliminating the errors due to the shifting of the zero point, as both right and left swings were always read and the average recorded. This also permitted us to detect open circuits and high resistances in the circuit, as when these occurred the swings were either very unsymmetrical or else were both on the same side of the zero point.

[3] White, W. P., *J. Am. Chem. Soc.*, 1914, xxxvi, 2011.

The condensation of moisture on the glass plate, on which the train stood, was a source of considerable trouble on humid days. For this reason several plates were in use and were dried out over a hot plate just before use.

The cell holder *C* consisted simply of a bottle fitted with a two hole rubber stopper through one hole of which was passed a tube terminating at its upper end in a funnel, *H:* in the other hole there was a tube ending in a fine capillary on which the cell was impaled in the

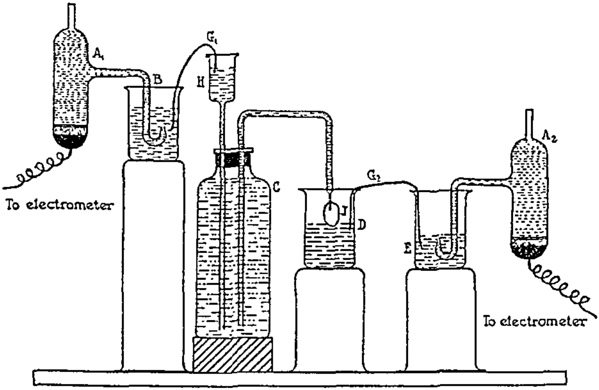

FIG. 2. Showing the manner in which the cell, *J*, is connected to the electrometer. The bottle, *C*, and the capillary on which *J* is impaled are filled with artificial sap.

manner indicated in Fig. 2. In use the bottle and tubes were filled with artificial sap, thus affording a connection to the inside solution of the cell. The sap in *H* was allowed to stand at such a level as to produce 2 to 3 inches of hydrostatic pressure at the tip of the capillary. In this way the cell was kept inflated. It is worthy of note that no leak occurred at the puncture at this or even at considerably higher pressures in the case of those cells which continued to live after being impaled. It will be seen that the cell in this arrangement hung free

from the tip of the capillary. No support was given it from below. This arrangement proved to be very satisfactory. Early in the work a considerable number of cells were lost by being shaken from the tip when the bottle was being moved. As our technique improved the losses from this cause were reduced to negligible proportions. However it was desirable to devise some method of supporting the cell from below, especially for prolonged measurements where the potential difference was to be measured from day to day for some time. Under these circumstances considerable handling of the cell holder and consequent shaking of the cell could not be avoided. To meet this situation the arrangement illustrated in Fig. 3 was devised.

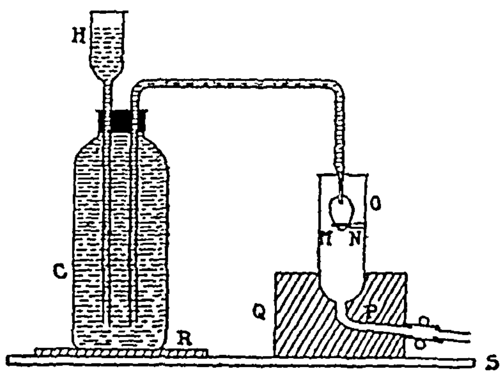

FIG. 3. Shows the ring *MN*, used to support the cell.

The cell holder *C* was as described above. The support for the bottom of the cell consisted simply of a glass ring, *M*, made of a 1 mm. glass rod, the opening of the ring being 3 to 5 mm. in diameter. The ring was sealed to a short piece of glass rod *N* and this in turn was sealed to the wall of the glass tube *O* (35 mm. in diameter) in such a way that the ring lay in a horizontal plane near the center of *O*. The tube *O* was drawn out at the lower end and the narrow tube *P* sealed on to form a drain. This was closed by means of a rubber tube and pinch-clamp. This part of the apparatus was imbedded in the manner indicated in the block of paraffin *Q* and this in turn was attached to the glass plate *S*. *C* was held in position on the plate by a thin layer of paraffin *R*. *C* and *O* were fixed to the plate in such a manner that the cell rested on the ring *M* but was not under enough pressure to

cause the cell to collapse even slightly. This adjustment was somewhat troublesome. It was accomplished in the following way. With C free from the plate, the cell was impaled. Then the capillary tube was moved up or down in the rubber stopper slightly until, when C was placed on the thin layer of paraffin R, the cell did not quite touch the ring. The layer of paraffin was then melted by heating the glass plate from below and in most cases the bottle settled so as to bring the bottom of the cell into the required position just on the ring. When

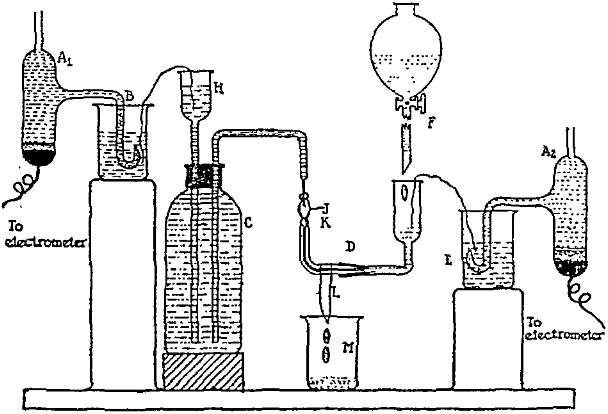

FIG. 4. Shows the manner of applying a flowing junction at K: the current of solution from F overflows at K and is carried by a strip of filter paper, L, to the waste beaker, M.

the paraffin hardened on cooling it held C in position. This part of the chain, cell and support, which was also the solution holder, was a fairly rigid unit. It could be moved without shaking the cell. In practice a number of these units were made up so several experiments could be carried out simultaneously.

In both the chains described above the solution applied to the outside of the cell was stationary. In some experiments (particularly those where the external solution differed from the internal) a flowing

junction was used. The apparatus with this modification introduced is illustrated in Fig. 4. In this train the beaker *D* of Fig. 2 was replaced by the U-tube *D* of Fig. 4. This U-tube (built in two parts with a ground glass joint for ease in emptying and cleaning) consisted of a wide right arm and a capillary left arm, the end of the thick walled capillary *K* being ground roughly plane. The required solution was dropped from the funnel *F* into the wide arm at such a rate that a slight head was maintained on the right-hand side of the U-tube. The rate of flow from the U-tube was of course controlled by the length and bore of the capillary left arm. The drops of solution overflowing from *K* were led into the waste beaker *M* by means of the strip of filter paper *L*. *C*, the cell holder, was placed in such a position that the cell *J* did not quite touch the drops rising in *K*. Then when *J* was lowered very slightly the liquid was drawn up and held in contact by surface tension. In this way a current of solution came into contact with a small area of the cell. The connection between *B* and *H* and *D* and *E* was made as before by means of damp strings. A further modification permitting the use of a supporting ring with the flowing junction is shown in Fig. 5.

The operation of impaling the cell so that it would cling to the capillary and live was not difficult provided certain conditions were fulfilled. It was almost essential that the capillary should be very fine, with sides as nearly parallel as possible, and that the glass at the tip should be very thin. It was also found advantageous to break off the tip of the capillary on a slant. In impaling the cell a brisk stream of liquid was forced out of the tip by blowing in at *H* and the cell was then slipped on to the capillary with one quick motion. Proceeding this way very few of the cells showed any sign of leakage or loss of their original contents. In some cases a small amount of natural sap was drawn up into the capillary before impaling the cells. This was to prevent the possibility of the cell sap being contaminated by artificial sap. However, as no ill effect was noticed in the experiments where this precaution was not taken it was abandoned.

Valonia macrophysa, as it occurs in Bermuda, is found in clumps containing many cells. Many of the cells used had one or more buds but in other cases they were lacking. However, in all the experiments impaling was carried out in such a way that the buds did not come

into the circuit (Fig. 6). The cells employed varied from 1 to 3 inches in length.

The object of the experiments was to measure the potentials in the circuit indicated by the dotted line in Fig. 1. But since the cell wall was imbibed with sea water and was wet on the outside the possibility of an electrical leak at the puncture must be considered. Such a leak would be equivalent to connecting the inside and outside of the cell by the dotted line in Fig. 7. If the liquid leak at the puncture were marked we should expect the electrical leak to be serious and as a result there should be no measurable potential difference. If, *on the other hand*, the leak were very slight then we should

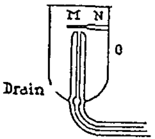

FIG. 5.

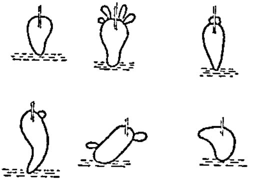

FIG. 6.

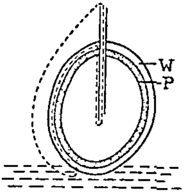

FIG. 7.

FIG. 5. Shows the manner of applying a flowing junction (as in Fig. 4) when the cell is supported by a ring, *MN*.

FIG. 6. Shows the manner of impaling cells on the capillary.

FIG. 7. The dotted line shows the short circuit in case of a leak at the capillary. *W*, cell wall; *P*, protoplasm.

expect considerable resistance in this short circuit and as a result there should be a measurable potential difference in the circuit through the wall. This would be less than the actual potential difference between the internal and external solutions. Now as has been pointed out above no liquid leak could ever be detected in the case of those cells which lived. On the other hand, considerable information was collected concerning the electrical leak at the puncture during the course of over 100 preliminary experiments. These experiments will first be described and discussed and then their bearing on the question of the electrical leak will be considered.

These experiments were carried out with the object of finding out whether a living cell always gave a positive potential against its own sap, and, incidentally, against sea water. At this point it is desirable to explain a convention adopted in this paper: the potential stated is always that of the inside of the cell. Thus 28 millivolts plus means that the inside of the cell was 28 millivolts more positive than the external solution bathing the cell, *i.e.* the positive current tends to flow from the capillary through the electrometer to the solution in contact with the outside of the cell. In these preliminary experiments the cell was pierced and the potential difference at once measured against sap and against sea water. The cells were then allowed to stand in sea water overnight, impaled on the capillary, and the two measurements were repeated the next day. The cells were again returned to sea water overnight and further measurements made. This was repeated as long as the cell lived, the intervals between measurements being gradually increased. The criterion for death in these experiments was a change in the arrangement of the chloroplasts, as observed with the naked eye or with a small magnifying glass. The cells immediately after being impaled could be divided into two groups: (*A*) the internal solution was positive against sea water outside and more positive against artificial or natural sap outside; (*B*) the internal solution was either at the same potential or very slightly negative to sea water, and at the same potential as the external sap. After standing in sea water overnight, cells of the *A* group either gave a positive potential difference against sea water outside and a still more positive potential difference against natural and artificial sap: or else the internal solution was at the same potential or slightly negative to the external sea water, and at the same potential as the artificial sap. In the former cases the cells were definitely alive. In the latter cases the cells were all dead: the chlorophyll layer was interrupted or completely broken up. Cells of the *B* group, after standing in sea water overnight, gave a positive potential difference against sea water outside and a greater positive potential difference against natural or artificial sap or else they gave little or no negative potential difference against sea water outside and no potential difference against sap outside. As in the case of the *A* cells on the 2nd day, those cells which were positive against sap and sea water were alive

while those which were not positive were dead. Many of the cells lived several days and some of them a month or more. From these experiments it seemed fair to conclude that normally the internal solution is positive to sea water outside and still more positive to sap.[4]

There was no difficulty in accounting for the behavior of those cells of Group *A* which on the 2nd day gave no measurable potential differences since they were dead, but with regard to those cells of Group *B* which on the 2nd day gave positive potentials against sea water and sap the hypothesis was advanced that the leak around the capillary had been eliminated by the formation of an "electrical seal" and consequently the disturbing short circuit had disappeared. To investigate this electrical seal a further series of experiments was carried out.

In these experiments a number of cells were impaled and the potentials of the internal solution were at once measured against sea water, first with the cells just in contact with the sea water and then with the cells completely immersed. Before being measured the cells were allowed to stand in sea water impaled on the capillary for periods ranging from 32 hours to 11 days. It was anticipated that where the liquid leak was considerable the measured potential in both cases would be zero or very small and that where the leak was less there would be a measurable plus potential with only the tip immersed and that this would fall almost or quite to zero with the whole cell immersed. On the other hand, if a cell could so cling to the capillary as to offer very great resistance to the passage of current at the puncture, we should anticipate positive potentials in both readings and the

[4] With cells of Group *A* the addition of a small amount of a toxic non-electrolyte to the external solution causes marked characteristic changes in the potential difference, the potential changing, after a few seconds, to that observed with cells known to be dead; while with cells known to be dead already, addition of chloroform or formaldehyde has no effect on the potential difference. This is an especially useful criterion of injury in cases where the applied solution is itself toxic. Cells killed by such solutions do not always show obvious signs of injury at once, and we may often be in doubt as to whether a measured potential is characteristic of the living or the dead cell. Addition of a small quantity of chloroform or formaldehyde solves the problem: if no change in the potential difference occurs, we know that the cell was dead; if a change does occur, the cell must have been alive, although possibly injured.

more perfect the seal at the capillary the more closely they should agree. As Table I shows, all those cells which were freshly impaled gave smaller potential differences when immersed than when just touching the sea water. On the other hand, those cells which remained alive in sea water for long periods gave nearly the same result with both kinds of measurement.

Obviously the error due to a short circuit can be almost completely eliminated by allowing the cell to stand in sea water impaled on the capillary. In the following work this was always done and before each experiment the seal of each cell was tested by measuring the potential difference against sea water both when the tip alone was in the solution and when the whole cell was immersed. Only those cells which gave satisfactory agreement of these two measurements were used in further experiments. It was observed in the case of those cells which had a good seal that a ring of dark material had been deposited at the puncture. That a cell is capable of healing itself to the extent of filling punctures is proved by our experience with certain cells which accidentally fell from the capillary into sea water. In several of these cases the cell continued to live and became as turgid as an unpunctured cell. The puncture was seen to be filled with a dark material which projected from the wall perhaps as much as half a millimeter and had the appearance of a tiny solid black plug. In a few other cases

TABLE I.

Cell No.	Time on capillary before measurement	Potential difference. Only tip immersed	Potential difference. Whole cell immersed
1	Newly impaled	9.3 plus	3.2 plus
2	" "	6.5 "	1.0 "
3	" "	3.3 "	0.7 minus
4	" "	7.2 "	0.4 "
5	8 days	5.2 "	4.9 plus
	9 "	4.4 "	4.4 "
6	11 "	4.6 "	4.0 "
	12 "	5.8 "	5.9 "
7	5 "	4.6 "	5.0 "
	6 "	4.6 "	4.8 "
8	32 hrs.	4.5 "	4.9 "
9	6 days	4.5 "	5.1 "

where a very fine capillary was used the capillary became plugged. The cell on the capillary became as hard as an unpunctured cell.

Possible sources of error due to diffusion potentials in the strings, stray currents, lack of balance between the calomel electrodes, etc., were found to be too small to merit consideration. At frequent intervals C was removed from the chain and a direct connection was made from B to D by means of string G_1 (Fig. 2). The observed potential difference was always either zero or at the worst slightly negative on the left.

The experiments were carried out in Bermuda at room temperature which varied from 15° to 20°C. But the variation in any one set of experiments was not over 3°C.

When the potential difference is measured in this way we have the chain:[5] sap | protoplasm | sap. Since this chain appears to be symmetrical we should expect its electromotive force to be zero. We find, however, a value of 14.5 millivolts,[6] the inside of the cell being positive to the outside (*i.e.* the positive current tends to flow through the electrometer from the capillary to the solution in contact with the outside of the cell). We must therefore conclude that the proto-

[5] The cell wall is omitted since it does not seem to play a part in the results now under consideration. For a discussion of the rôle of the cell wall and the underlying principles governing the production of potential differences in these cells see Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83.

[6] This is the average of 100 determinations giving the following figures:

Minimum potential difference	4.3	millivolts
Maximum " "	38.0	"
Mean of 100 experiments	14.5	"
Average deviation from the mean	3.5	"
Probable error	0.3	"
" " expressed as per cent of the mean	2.1	

In an earlier paper by one of us (Osterhout, W. J. V., *J. Gen. Physiol.*, 1924–25, vii, 561) it was stated that the arrangement shown in Fig. 1 gives very little potential difference when sea water is in contact with the outside of the cell. More recently Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., *Carnegie Institution of Washington*, 1925-26, xxv, 248) have obtained a similar result. There is no assurance that an electrical seal was formed in these experiments. When such a seal is obtained we find a potential difference of about 5 millivolts.

plasm is not symmetrical and this raises the question of its probable structure.

It has been suggested by one of us that the protoplasm may be composed of layers, the simplest assumption being that the outer surface, X, and the inner surface, Y, are probably non-aqueous, while between them lies an aqueous layer, W. If we adopt this as a working hypothesis, we may consider that we have the chain

$$\text{sap} \mid X \mid W \mid Y \mid \text{sap}$$

and that X and Y are unlike, which would give an unsymmetrical chain. In this way we might explain the source of the observed potential difference since it has been shown by Cremer,[7] and by Beutner,[7] that when two different liquids immiscible with water are brought into contact with aqueous solutions so as to make a chain similar to the one we are discussing, a potential difference may result. Mond[8] obtained similar results with chains consisting of acid and alkaline gelatin or of euglobin and casein.

It would therefore appear that the inner and outer surfaces of the protoplasm are quite different. It has recently been suggested by one of us that this must be the case since certain substances are apparently able to penetrate the outer surface but not the inner.

These views are in line with experiments[9] on the marine alga *Griffithsia* which indicate a difference in behavior of the two surfaces, and in harmony with the earlier work of de Vries[10] if his results may be interpreted to mean that the inner and outer surfaces do not act alike.

It may be added that Höber,[11] in discussing the current of injury in muscle, mentions the possibility that the inner and outer surfaces of the protoplasm may be unlike. But since there is no vacuole in such cells it is evident that the situation is quite different from that

[7] *Cf.* Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

[8] Mond, R., *Arch. ges. Physiol.*, 1924, cciii, 247.

[9] Osterhout, W. J. V., *Science*, 1913, xxxviii, 408.

[10] de Vries, H., *Jahrb. wissensch. Bot.*, 1885, xvi, 465. See also Küster, E., *Ber. bot. Ges.*, 1909, xxvii, 589; *Arch. Entwcklngsmechn. Organ.*, 1910, xxx, pt. 1, Festschrift für Wilhelm Roux, 351; *Z. Bot.*, 1910, ii, 689.

[11] Höber, R., Physikalische Chemie der Zelle und der Gewebe, 6th edition, Leipsic, 1926, 732.

described in this paper. The difference of which Höber speaks can hardly be between X and Y (as discussed above), but perhaps rather between the inner and outer surfaces of X.

If it is true that in a thin layer of protoplasm the two surfaces may be so different, it is evident that our ideas regarding the factors that determine the structure of protoplasm are in need of revision. The subject deserves further investigation and will be discussed in subsequent papers.

SUMMARY.

The protoplasm of *Valonia macrophysa* forms a delicate layer, only a few microns in thickness, which contains numerous chloroplasts and nuclei. The outer surface is in contact with the cell wall, the inner with the vacuolar sap. As far as microscopic observation goes, these two surfaces seem alike; but measurements of potential difference indicate that they are decidedly different. We find that the chain

sap | protoplasm | sap

gives about 14.5 millivolts, the inner surface being positive to the outer. In order to explain this we may assume that the protoplasm consists of layers, the outer surface, X, differing from the inner surface, Y, and from the body of the protoplasm, W. We should then have the unsymmetrical chain

sap | X | W | Y | sap

which could produce an electromotive force.

If the two surfaces of such a very thin layer of protoplasm can be different, it is of fundamental significance for the theory of the nature of living matter.

ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume is appearing simultaneously with Volumes IX and X. Number 1 of this volume will contain a biography of Dr. Loeb. It is to appear after Number 6, and the page numbers will be roman numerals. The publication of this volume began September 18, 1925.

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THE JOURNAL OF GENERAL PHYSIOLOGY

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ACID PENETRATION INTO LIVING TISSUES.

By NELSON W. TAYLOR.

(From the School of Chemistry, University of Minnesota, Minneapolis.)

(Accepted for publication, May 23, 1927.)

The factors governing acid penetration into living tissues have never been clearly stated and we are therefore in ignorance as to the underlying physicochemical basis of the sour taste and allied phenomena. In this paper the writer presents quantitative evidence which he believes, shows: first, that acid penetration through a membrane occurs either in the form of the undissociated molecule or by the simultaneous passage of H^+ ion and anion; secondly, that the sourest acids are those which penetrate most rapidly; and finally that the relative rates of penetration of a series of acids are determined by the well known physicochemical laws of adsorption.

It was early realized that solutions of equal stoichiometrical acid concentration were not equally sour. Thus, for example, the threshold concentrations of HCl and CH_3COOH which just excite the sour taste are 0.001 N and 0.003 N respectively. Becker and Herzog (1907) state that the intensity of the taste sensation occasioned by acids of equal normality decreases in the following order: hydrochloric, nitric, trichloroacetic, formic, lactic, acetic and butyric. Paul (1922) in a series of papers on the physical chemistry of foodstuffs has determined the concentrations of acids which are equally sour with certain standard HCl solutions. Taking 0.005 N HCl as a reference he finds the order of increasing acid concentration is as follows: tartaric acid (lowest concentration therefore most sour), hydrochloric, acetyl lactic, lactic, acetic, carbonic. It is noteworthy that the two acids:—tartaric and acetyl lactic, which have practically identical dissociation constants (9.7×10^{-4}) are equally sour only when the former is 0.004 N and the latter 0.0059 N. Data such as this show that some other factor beside acid concentration plays a part in determining sourness intensity.

That this factor is not purely hydrogen ion concentration (or activity) must be evident from the data which I have obtained from experiments carried out in this laboratory. My experimental procedure consisted at first in giving 5 cc. samples of a number of concentrations of formic acid in the vicinity of the threshold concentration to fifteen people and thus determining the minimum concentration which was definitely sour. Astringent sensations are detectible in weaker solutions. A 5 cc. sample placed in the mouth is better than a few drops, as used by some investigators, because the former represents more nearly the average condition in the mouth rather than that of a single spot on the tongue. The mouth was rinsed with boiled distilled water between each test. Having determined the sour threshold concentration for formic acid, solutions of other acids were taken and that concentration of each acid chosen which was indistinguishable from the standard formic acid solution. On the average ten people passed judgement on each of these solutions. In cases where the acid had an odor the nose was held during each test. This effectively excluded the odor and restricted the judgment purely to the question of sourness. All solutions were at room temperature (22°–25°) and had been prepared from stock acid solutions of known concentration.

The hydrogen ion activities of these solutions were determined electrochemically by means of cells of the type Hg | HgCl, KCl saturated | acid | H_2, measurements being made at 20°C. Assuming that in these dilute solutions the activity coefficient of the H^+ ion is unity, we may write "concentration" instead of "activity." The data are recorded in Table I.

Since the values in Column 2 of this table differ from acid to acid it is evident that sourness is not purely a function of the stoichiometrical acid concentration. Further, since the hydrogen ion concentrations are not the same for the several acids we may conclude that this is not the determining factor. What then is the explanation of these results?

It occurred to the writer to compute the ratio of the concentrations of the undissociated part of each acid outside and inside the taste bud. The former concentration can readily be calculated from the data above, and are recorded in Column 2 of Table II. In the case of the

dibasic acids having anions $A^=$ and HA^- the concentration of the undissociated acid "outside" (H_2A) has been computed from the expression: Total acid concentration in mols $= H_2A\left(1 + \frac{K_1}{H^+} + \frac{K_1K_2}{(H^+)^2}\right)$

TABLE I.

Threshold Concentrations for Sour Taste.

	Total acid concentration	H^+ ion concentration
Formic	0.0018 N ± 3 per cent	0.00055
Acetic	0.0028 N ± 6 " "	0.00028
Butyric	0.0035 N ± 6 " "	0.00027
Valeric	0.0037 N ± 5 " "	0.00015
Oxalic	0.0020 N ± 8 " "	0.00116
Succinic	0.0032 N ± 7 " "	0.00034
Glutaric	0.0045 N ± 5 " "	0.00034
Lactic	0.0028 N ± 5 " "	0.00177
Tartaric	0.0022 N ± 7 " "	0.00070

TABLE II.

Concentration Gradients of Equally Sour Acids (from Threshold Concentration).

Acid	Concentration of undissociated acid Outside (a)	Relative concentration of undissociated acid Inside (b)	Ratio a/b when formic = 1
Formic	.00125	1	1
Acetic	.00252	11.8	0.17
Butyric	.00323	14.75	0.17
Valeric	.00355	13.4	0.21
Lactic	.00103	1.53	0.54
Oxalic	.0000285	0.0036	6.3
Succinic	.00134	3.2	.34
Glutaric	.00196	4.3	.38
Tartaric	.000444	0.22	1.60

where K_1 and K_2 are the first and second dissociation constants respectively. The inside concentration can be calculated, relatively but not absolutely, from the dissociation constants of the acids if we assume that the production of a given degree of sourness is due to the establishment within the cells of the taste bud of a definite hydrogen

ion concentration. Thus, for example, if two solutions of acetic acid and formic acid have the same hydrogen ion concentration; the concentrations of the undissociated parts stand together in the ratio $\frac{(CH_3COOH)}{(HCOOH)} = \frac{K_{\text{formic}}}{K_{\text{acetic}}} = \frac{2.14 \times 10^{-4}}{1.8 \times 10^{-5}} = 11.8$. In the case of dibasic acids the first dissociation constant K_1 was used for this computation and the effect of K_2 neglected, since it is impossible with the data available to take its effect into account quantitatively. The error will, however, be fairly small. These values are listed in Column 3 of

TABLE III.

Concentration Gradients of Equally Sour Acids, from Data by Paul and Bohnen.

Acid	Total acid concentration	H^+ ion concentration	Concentration of undissociated acid Outside (a)	Relative concentration of undissociated acid Inside (b)	Ratio a/b when formic = 1
	$\times 10^{-4}$M	$\times 10^{-4}$M	$\times 10^{-4}$M		
Formic	7	3	4.0	1.00	1
Acetic	22	1.9	20.1	11.8	0.43
Propionic	33	2.56	30.4	14.75	0.53
Butyric	5	0.96	4.0	14.75	0.068
Monochloroacetic	12	8.0	4.0	0.138	7.3
Monobromoacetic	11	7.2	3.8	0.155	6.1
Lactic	23	5.0	18	1.55	2.9
Succinic	8	2	6	3.2	0.48
Malic	8	4	4	.53	1.9
Tartaric	4	3	1	0.22	1.1
Carbonic	53	0.4	52.6	705.	0.019

Table II under the heading "Relative concentration of undissociated acid inside." The ratios of the numbers in Columns 2 and 3 are found in Column 4, and these ratios may be taken to represent the relative gradients or driving forces which are necessary to cause the several acids to penetrate the tissues to a comparable degree (*i.e.* producing equal H^+ ion concentration inside).

Paul and Bohnen (see Paul, 1922) have obtained limiting concentrations for various acids which taste sour. Their results are given in Table III, Columns 2 and 3. I have calculated the quantities in the other columns from their experimental data.

Before discussing in detail the physicochemical basis for the different behavior of these acids in regard to taste excitation, I wish to draw attention to some experiments on the *rate* of acid penetration. Studies on rates have been made by Haas, Jacobs, Collett, Brooks, Crozier, Osterhout, Gompel and others. Crozier (1916) has measured the rate of penetration of various acids into the mantle fold of *Chromodoris zebra.* This tissue contains a natural indicator which changes color

TABLE IV.

Concentration Gradients of Acids, Penetrating Chromodoris Tissue and Producing a pH = 5.6 in 20 Minutes.

Acid	Total acid concentration	H^+ ion concentration	Concentration of undissociated acid *Outside* (a)	Concentration of undissociated acid *Inside* (b)	Ratio a/b when formic = 1
	$\times 10^{-4}M$	$\times 10^{-4}M$	$\times 10^{-4}M$	$\times 10^{-3}M$	
Formic	33.3	7.5	25.8	2.95	1.00
Acetic	188.8	5.8	183	34.7	0.60
Propionic	142.5	4.5	138	43.5	0.36
Butyric	93.4	3.6	89.8	43.5	0.24
iso-Valeric	38.4	2.4	36	39.4	0.10
Lactic	52.1	7.9	44.2	4.51	1.12
Benzoic	53.1	5.3	47.8	10.5	.52
Salicylic	35.1	14.4	20.7	.63	3.76
Succinic	84.7	7.1	77.6	9.56	0.93
Malic	74.0	15.3	58.7	1.58	4.26
Tartaric	66.6	21.0	45.6	.65	8.00
Oxalic	20.0	19.05	0.95	0.016	6.5
Malonic	52.6	22.1	30.5	0.39	8.8
Monochloroacetic	39.2	18.1	21.1	.41	5.95

from blue to pink at a pH of 5.6. Crozier's observations cover several concentrations of a dozen or more acids. I have replotted his curves of penetration time versus normal dilution and have determined graphically the concentrations of the various acids necessary to penetrate the tissue and produce the color change in a given time, for example, 20 minutes. Table IV shows these concentrations along with calculated values of the equilibrium concentration of the undissociated acids outside the tissue (*a*), and inside the tissue (*b*) when

pH = 5.6. The last column of the table gives the ratio a/b, which is a measure of the gradient driving the acid through the cell membrane.

The data in this table are for a penetration time of 20 minutes. If 30 minutes or 40 minutes had been chosen the a/b ratios would of course be changed but the general results would be the same. That is, the same trend would appear for the fatty acids, and the hydroxy acids lactic and salicylic would require a larger concentration gradient (a/b) than the corresponding acids, propionic and benzoic respectively. This is evident from the plot of penetration time versus normal dilution because there is no crossing of corresponding curves in the plot in this time interval.

TABLE V.

Concentration Gradients of Acids Causing Retraction of Earthworm in 10 Seconds.

Acid	Total acid concentration	H^+ ion concentration	Concentration of undissociated acid Outside (a)	Relative concentration of undissociated acid Inside (b)	Ratio a/b when formic = 1
	$\times 10^{-3}$ M	$\times 10^{-4}$ M	$\times 10^{-4}$ M		
Formic	11	14.3	96	1.00	1.00
Acetic	32	7.6	312	11.8	.28
Propionic	22.5	5.6	219	14.8	.15
Butyric	23	5.7	224	14.8	.16
iso-Valeric	18	5.3	175	13.4	.14
Caproic	11	3.9	107	14.8	.075
Caprylic	1	1.1	9	14.8	.006
Monochloroacetic	8.4	29.1	55	.138	4.17
Dichloroacetic	5.8	52.5	5.5	.0042	13.5

Crozier (1917–18) has also studied the phenomena of sensory activation by acids in the earthworm *Allolobophora sp.* His method was to place an earthworm with one end in distilled water and the other in an acid solution and to measure the time of retraction of the worm from the acid solution. Again I have plotted his data and determined graphically the effective acid concentrations at fixed times, 10 seconds and 20 seconds, in which period the experimental accuracy seems to be greatest. Tables V and VI contain this material together with appropriate calculations by the writer. The gradients obtained from

Tables II to VI inclusive are collected in Table VII which allows a comparison to be made of the results of the different experiments. These are not absolute gradients but are relative, taking that for formic acid equal to unity. It should be quite evident that those acids which require the smallest gradient are those which penetrate the cell walls of the living tissue most easily.

In comparing the various sets of data attention should be focussed on the trends in the values for a series of acids rather than on the quantitative magnitude of the values for a given acid in the different experiments.

TABLE VI.

Concentration Gradients of Acids Causing Retraction of Earthworm in 20 Seconds.

Acid	Total acid concentration	H^+ ion concentration	Concentration of undissociated acid Outside (a)	Relative concentration of undissociated acid Inside (b)	Ratio a/b when formic = 1
	$\times 10^{-3}M$	$\times 10^{-4}M$	$\times 10^{-4}M$		
Formic	6	10.3	49.7	1.00	1.00
Acetic	15	5.1	145	11.8	25
Propionic	11	3.9	107	14.8	.14
Butyric	9.5	3.6	91.4	14.8	.12
iso-Valeric	7	3.3	66.7	13.4	.10
Caproic	4 5	2.5	42.5	14.8	.058
Caprylic	0.8	1.0	7.0	14.8	.0094
Monochloroacetic	6.2	24.2	38	.133	5.5
Dichloroacetic	4.7	43.3	3.7	.0042	17.6

Evidence for Adsorption by Cell Wall.—The fatty acid series shows a well marked trend from formic to caproic, particularly in the experiments on penetration rate, but less clearly in the taste data where figures are available. It seems likely that the higher members of the series are more readily adsorbed by the tissues than are the lower members. The last column of Table VII contains Freundlich's results on the relative concentrations of these undissociated acids which are necessary in order that charcoal may adsorb the same quantity of acid in each case. Thus for example if a butyric acid solution is 6 per cent as concentrated as a formic acid solution, the same quantity

of either acid will be adsorbed by charcoal. The excellent agreement between these results and those on the living tissues suggests that the fatty acids are taken into such tissues by an *adsorption process.* These acids show the same behavior in their toxic action on Ciliate Infusoria. In a paper on this subject Collett (1919) has measured the

TABLE VII.

Relative Concentration Gradients of Undissociated Acids across Living Tissues under Comparable Sets of Conditions.

Acids	From taste data		From penetration rate (Crozier)			From equal charcoal adsorption
	Taylor	Paul and Bohnen (see Paul, 1922)	*Chromodoris*	*Allolobophora*		
			20 min.	*10 seconds*	*20 seconds*	
Formic	1.00	1.00	1.00	1.00	1.00	1.00
Acetic	0.17	0.43	0.60	0.28	0.25	.68
Propionic		0.53	0.36	0.16	0.14	.22
Butyric	0.17	0.068	0.24	0.16	0.12	.06
iso-Valeric	0.21		0.10	0.14	0.10	
Caprylic				0.075	0.058	
Caproic				0.006	0.009	
Bromoacetic		6.1				
Chloroacetic		7.5	5.95	4.17	5.5	
Dichloroacetic				13.5	17.6	
Lactic	0.54	2.9	1.12			
Benzoic			0.52			
Salicylic			3.76			
Succinic	0.34	0.48	0.93			
Malic		1.9	4.26			
Tartaric	1.60	1.1	8.00			
Oxalic	6.3		6.5			
Malonic			8.8			
Glutaric	0.38					
Carbonic		0.019				

time necessary for a series of acids of equal pH to stop the beating of one-half the cilia taken for the test. He found the order of decreasing toxicity to be for paramecium at pH 3.5; valeric, butyric, propionic, acetic, formic. The same order held for *Euplotes* at pH 3.5 and pH 4.0. These results entirely oppose the old-fashioned idea that the cell walls have little holes through which an acid molecule may

squeeze for we actually find that the largest molecules, caprylic and caproic, enter most easily and the smallest (*i.e.* formic) enters with the greatest difficulty.

Influence of Polar Groups.—The retarding effect of polar groups is evident. On the average the necessary concentration gradients are approximately 5 times greater for the hydroxy acid than for the simple acid. Compare lactic and propionic, salicylic and benzoic, tartaric, malic and succinic. A halogen atom seems to have even greater effect. Thus the average ratio of chloroacetic to acetic is about 15, and two chlorine atoms added to acetic acid produce more marked retardation than two hydroxy groups added to succinic. Bromoacetic is like chloroacetic. It is noteworthy that carbonic acid, which may be looked upon as hydroxy-formic, does not have a gradient 5 times greater than formic but on the contrary it is 50 times smaller. It would appear that anhydrous CO_2 entered the tissues rather than H_2CO_3. Carbon dioxide is very soluble in oily liquids. That this gas has a very high penetration velocity was shown by Jacobs (1920), by measuring the time necessary for acid solutions of equal pH to produce a blue to pink color change in the natural indicator in the flowers of *Symphytum peregrinum.* Carbonic acid produced a color change in about 2 minutes, benzoic and valeric require about 15 to 30 minutes, and butyric, acetic and salicylic take longer times in the order listed. Jacobs stressed the relation between speed of penetration and sourness in the case of CO_2. It would seem as if the specific action of CO_2 on the respiratory center is largely due to its rapid penetration of the tissues even though other weak acids may be present. It is to be noted that Jacobs found salicylic to be slower than benzoic which agrees with Crozier's results on *Chromodoris.* It is difficult to estimate accurately the concentrations of equally sour benzoic and salicylic acids because of the fact that the latter has a very well marked *sweet* taste. This very fact is an indication of low cell membrane permeability because it is well known that other sweet tasting substances, sugars and polyhydric alcohols (which contain many hydroxyl groups) can penetrate cell membranes only with great difficulty. Gallic acid (trihydroxy benzoic) also has its sour taste obscured by a sweet taste.

One may ask why the adsorbability of a substance determines its

rate of adsorption. Comparing formic and butyric acids we see from Freundlich's experiments on charcoal that the latter acid is attracted more strongly by the adsorbent, or in thermodynamic terms that the free energy decrease on adsorption is greater. If we make the reasonable assumption that the adsorption mechanism is the same for all members of a given homologous series it follows that the adsorption velocity will be greatest where the free energy decrease is greatest. *One would therefore expect to find that for a given penetration velocity successively smaller concentration gradients are necessary as one changes from formic acid to its higher homologs.*

The nature of the cell membrane probably determines the effects noted in this paper. It is well known that the fatty acids depress the surface tension of water in the order: valeric > butyric >propionic > acetic > formic. According to the Gibbs theorem this fact means that their tendency to leave the body of the aqueous solution and concentrate on the surface follows the same sequence—hence their relative tendency to leave aqueous solutions and penetrate an *oily membrane.* The polar hydroxyl group and the chlorine atom increase the water solubility of an organic acid, consequently such substitution products have less tendency to enter an oil film than would the simple acid. Finally, oxalic acid is much more water-soluble than butyric, an acid having about the same molecular weight. It even forms a stable hydrate $C_2H_2O_4 \cdot 2H_2O$. It is therefore much less likely to leave the aqueous solution and enter the membrane if the latter were of an oily character. There is of course a great deal of independent evidence as to the fat or lipoid nature of cell membranes.

Stereochemical Effects.

Another factor of very great importance in connection with acid penetration of tissues is the optically active character of the acid. It is well known that a large proportion of the chemical constituents of living tissues is made up of asymmetrical material. Pasteur was the first to separate an equimolecular mixture of *d*-acid and *l*-acid by addition of an optically active base, for example, quinine. The solubilities and other properties of the compounds so formed differ much more from each other than if an inactive base had been used. Consequently, since much tissue material is asymmetric, *d*- and *l*-acids

should be quite different in their physiological action. The fungus *Penicillium glaucum* grows readily in fumaric acid but only very slowly in a maleic acid solution. The reverse occurs in the case of *Lupinus albus* L., discovered by Kahlenberg and True (1896). Winther (1895) reviewed the literature on the action of organisms and reported that *d*-tartaric, *l*-lactic, *l*-mandelic, *d*-glutaminic and *l*-ethoxysuccinic acids have been prepared by the action of *Penicillium glaucum* on solutions of the racemic acids, showing that these optical acids are taken into the tissues of the fungus and metabolized less readily than their optical isomers. Fumaric acid has a pure acid taste, maleic is in time biting and nauseous; citraconic acid is bitter, mesaconic acid is sour. Quantitative experiments like those of Crozier on penetration rate would be of great interest in this connection and would probably lead to a clearer understanding of the chemical as well as physical nature of cell membranes.

Penetration Mechanism.

The writer has stressed the relation between experiments on penetration *velocity* and degree of *sourness* of various acids. Why is it that the excitation of a taste sensation seems to depend on a velocity factor? Let us consider an electrical analogy. If a copper wire placed in a magnetic field is suddenly jerked out a current is developed in the wire for a short time and may be observed with a ballistic galvanometer. On the other hand if the wire is removed slowly from the field only a very feeble current is developed. Here the time factor plays the major rôle. It would seem that the nervous impulse sent to the brain which we interpret as a sour taste requires for its excitation a rapid change in H^+ ion concentration in the taste bud. For this reason it is unlikely that data applicable for testing any theory of the sour taste can be obtained from experiments which measure H^+ ion or acid concentrations at *equilibrium conditions*. As an illustration of this latter type of experiment we have the work of Smith (1925) on the action of acids on cell division. "In these experiments the eggs were left in the solutions for one and a half to several hours, the time necessary to attain equilibrium between cells and fluid cannot influence the results, and has only theoretical significance."

All the intracellular effects noted in this paper have been considered

to be due to H^+ ion. This does not mean that H^+ ion alone has penetrated the cell walls leaving the anion outside because such a process would immediately build up an opposing electrostatic potential. There are, however, two ways by which H^+ ion might enter the cell interior. The first of these is by the simultaneous exit of another positively charged ion such as K^+ ion or Na^+ ion, comparable to the well known Cl^- ion—HCO_3^- ion exchange between red blood corpuscles and plasma during the respiratory cycle. If this alone were the determining factor we should find no differences between the sourness of acids of equal pH. On the other hand we find great differences, and further there is much evidence that Na^+ ions and K^+ ions do not pass very freely through cell membranes. We may therefore exclude this process in most cases. The second process is that as the H^+ ions are adsorbed into the cell membrane the electric charge so developed would attract the anion of the acid into the membrane as well, and thus the pair of ions would gradually penetrate into the cell interior. Such an assumption would seem to be necessary in the case of hydrochloric acid, which is of course sour and is able to penetrate many living tissues, and yet is considered to be completely ionized in accordance with the modern theory of strong electrolytes. We cannot here postulate that the undissociated HCl molecule is the only molecular species capable of penetration, because there is practically no HCl as such present. In order to be perfectly consistent in our treatment of weak organic acids and completely ionized inorganic acids we must assume that the former may also penetrate the membrane by the simultaneous passage of H^+ ion and anion. However, this mode of passage cannot be distinguished, electrically or thermodynamically, from the passage of undissociated molecules, since an ion pair will be neutral and since a temporary association of H^+ ion and anion during the penetration may be looked upon as a state of molecule formation. For solutions of equal pH the concentration gradient necessary to establish a given pH in the cell interior in a given time will therefore be determined by the ability of the anion to penetrate the cell membrane. The effects of polar groups on this phenomenon and the behavior of an homologous series have already been noted. Osterhout and Dorcas (1925) have studied the factors governing the penetration of H_2S and CO_2 into *Valonia macrophysa*. Their results are not inconsistent with the mechanism postulated in this paper.

SUMMARY.

The threshold concentrations for sourness of nine acids have been determined with an accuracy of about 8 per cent, and the H^+ ion concentration of these acids measured.

Calculations have been made of the relative concentration gradients of the undissociated acid across the cell membrane for a series of acids having equal sourness and also for a series of acids having equal penetration velocity as determined from experiments by Crozier on *Chromodoris* and on *Allolobophora*. For solutions of equal pH a high degree of sourness has been found to be associated with a high penetration velocity of the undissociated acid or of the anion. A comparison of these gradients with the results of adsorption experiments on charcoal indicates that the acids are taken into the tissues by an adsorption process.

Polar groups such as OH and Cl and Br are found to have a very marked effect in reducing the ability of organic acids to penetrate living tissues.

The important rôle of optical activity of the acids in determining their physiological action has been noted.

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MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

V. The Antagonism of Cations in Their Actions on the Protoplasm of Amœba dubia.

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By means of the micrurgical technique (1) combinations of salts in mutually antagonistic concentrations can be injected directly into a cell. This procedure, together with the immersion of cells in mixtures of the salts, permits the determination of the antagonistic action of such substances in their effect on the internal protoplasm and on the plasma membrane.

In the first paper (2) of this series it was demonstrated that NaCl and, to a less extent, KCl, tend to disperse the plasmalemma. $MgCl_2$ and $CaCl_2$, on the other hand, have no such disruptive action. It was also shown that NaCl and KCl increase the fluidity of the internal protoplasm but that $MgCl_2$ and $CaCl_2$ tend to solidify the internal protoplasm. Because of the variation in the degree of the effect of NaCl and of KCl it was considered of interest to study the action of a third salt with a monovalent cation, *viz.* LiCl. LiCl was found to have a much greater dispersing effect on the plasmalemma and to fluidify the internal protoplasm even more markedly than NaCl. So intense is this action that when LiCl is injected into the ameba it diffuses rapidly through the protoplasm and, in strong concentrations, disperses the plasmalemma much more readily than does NaCl. The opposite effects of the salts of mono- and of bivalent cations were used in the experiments to be described as criteria for a study of antagonism between the chlorides of Li, Na, and K and those of Ca and Mg.

In each case the mixtures used were made by combining equal volumes of the solutions of each salt in twice the concentration of

the desired strength, so that the final mixture represented the actual concentration to be tested. For each salt a graded series of solutions was prepared varying in strength from an immediately lethal concentration to one which was non-toxic, and each of the series was in turn mixed with concentrations of the antagonizing salt which varied from

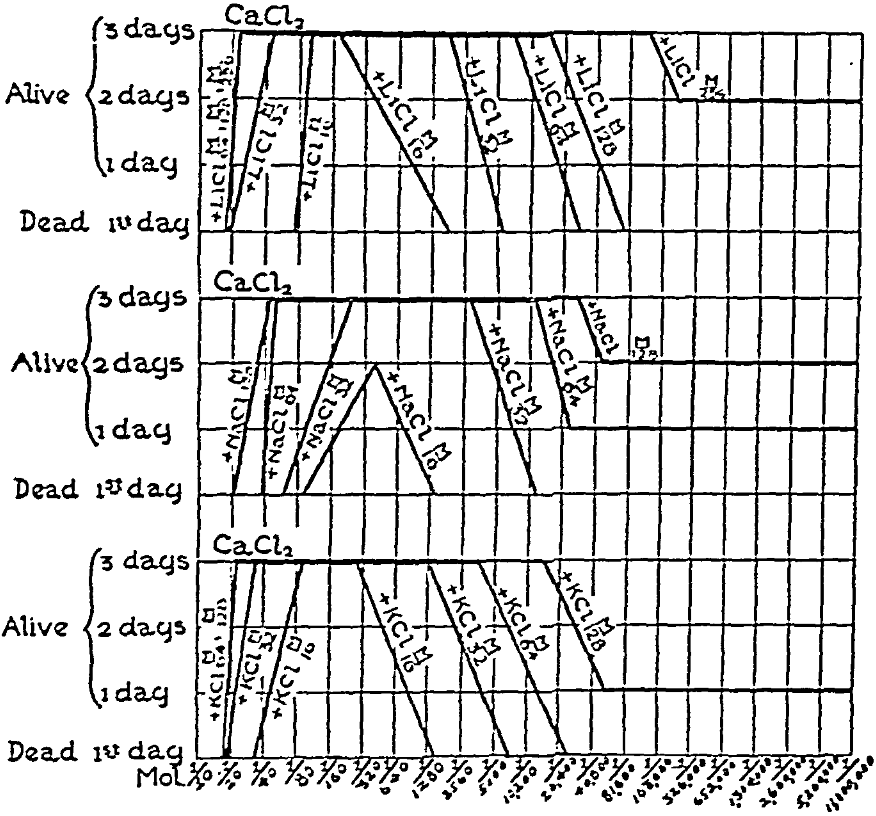

FIG. 1. Antagonism of $CaCl_2$ to toxic concentrations of LiCl, NaCl, and KCl in their action on the plasmalemma (immersion experiment). Abscissæ represent decreasing concentrations of antagonizing salt ($CaCl_2$). Each curve represents a toxic concentration of LiCl, NaCl, or KCl.

one exhibiting no antagonism to one in which the antagonizing salt itself was toxic.

Immersion Experiments.

Amebæ were immersed in varying concentrations of the binary mixtures of LiCl, NaCl, or KCl with $MgCl_2$ or $CaCl_2$. In these experi-

ments the antagonizing action of the salts of the bivalent ions upon the toxic effect of the salts of the monovalent ions was studied. The marked toxicity of LiCl, NaCl, and KCl upon immersed amebæ makes it impossible to study their antagonizing action on the toxic effect of $CaCl_2$ and of $MgCl_2$ in immersion experiments.

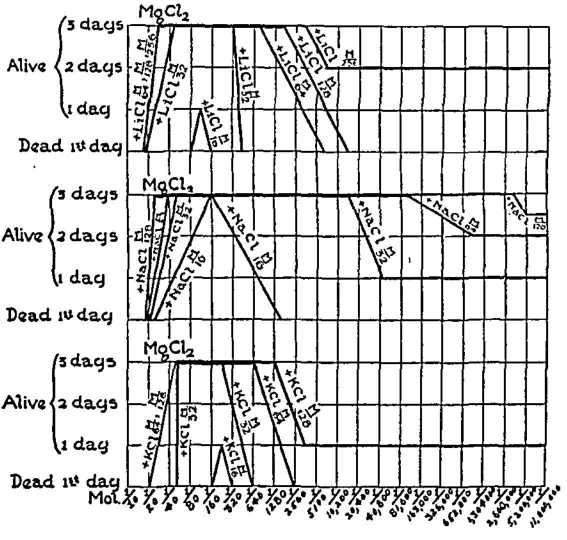

FIG. 2. Antagonism of $MgCl_2$ to toxic concentrations of LiCl, NaCl, and KCl in their action on the plasmalemma (immersion experiment). Abscissæ represent decreasing concentrations of antagonizing salt ($MgCl_2$). Each curve represents a toxic concentration of LiCl, NaCl, or KCl.

Fig. 1 shows the results obtained when amebæ are immersed in mixtures of toxic concentrations of LiCl, NaCl, or of KCl with an antagonizing concentration of $CaCl_2$. Because of the nature of the experiment this effect is exerted primarily on the plasmalemma. By comparing equivalent concentrations of the toxic salts it can be seen

that $CaCl_2$ antagonized LiCl sixteen times and NaCl four times a effectively as it did KCl (Fig. 3). $MgCl_2$ antagonizes LiCl (Fig. 3 three times and NaCl 3000 times as effectively as it does KCl (Fig. 3)

A comparison of the relative antagonizing power of $CaCl_2$ and c $MgCl_2$ shows that $CaCl_2$ antagonizes LiCl thirty-two times and KC

Toxic Salt		Antagonizing Salt $CaCl_2$	
Salt	Equivalent toxic concentrations	Effective concentrations	Relative effectiveness of antagonism
LiCl	$\frac{M}{256}$	$\frac{M}{25}$ — $\frac{M}{122,000}$	16
NaCl	$\frac{M}{128}$	$\frac{M}{45}$ — $\frac{M}{30,500}$	4
KCl	$\frac{M}{128}$	$\frac{M}{35}$ — $\frac{M}{7,600}$	1

Toxic Salt		Antagonizing Salt $MgCl_2$	
Salt	Equivalent toxic concentrations	Effective concentrations	Relative effectiveness of antagonism
LiCl	$\frac{M}{256}$	$\frac{M}{30}$ — $\frac{M}{3,800}$	3
NaCl	$\frac{M}{128}$	$\frac{M}{25}$ — $\frac{M}{3,600,000}$	3000
KCl	$\frac{M}{128}$	$\frac{M}{60}$ — $\frac{M}{1280}$	1

FIG. 3. Summary table of antagonism of $CaCl_2$ and $MgCl_2$ to toxic concentrations of LiCl, NaCl, and KCl in their action on the plasmalemma (immersion experiment).

six times as effectively as does $MgCl_2$. On the other hand, $MgCl_2$ antagonizes NaCl almost 115 times as effectively as does $CaCl_2$.

The outstanding feature of these immersion experiments is the marked antagonism of $MgCl_2$ to the toxic action of NaCl on the

plasmalemma. Figs. 1 and 2 indicate that the upper limit of antagonism is the toxic concentration of the antagonizing salt itself. However, with the higher concentrations of the salts the combined concentrated solutions of the two salts are more rapidly toxic than either one alone.

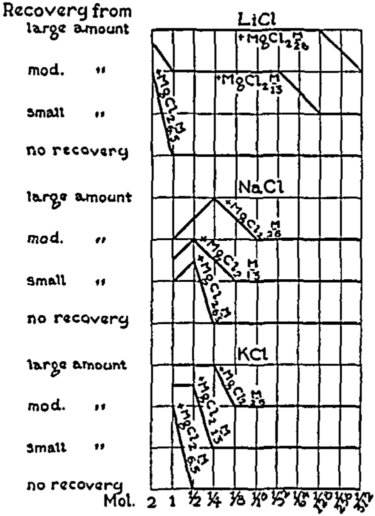

Fig. 4. Antagonism of LiCl, NaCl, and KCL to toxic concentrations of $MgCl_2$ in its action on the internal protoplasm (injection experiment). Abscissæ represent decreasing concentrations of antagonizing salts (LiCl, NaCl, and KCl). Each curve represents a toxic concentration of $MgCl_2$.

Injection Experiments.

In the injection experiments the salts of both the monovalent and bivalent cations can be used either as toxic agents or as antagonists. The antagonizing action of NaCl and KCl against the solidifying and pinching off effect obtained when $CaCl_2$ is injected into the ameba has

been considered previously (2). LiCl has a similar antagonizing action. LiCl, NaCl, and KCl have the same power of antagonizing the solidifying action of $CaCl_2$ in all concentrations used in these experiments, except in that of M/1. In this concentration, LiCl and KCl have twice the antagonizing power of NaCl.

The antagonizing power of LiCl, NaCl, and KCl against toxic concentrations of $MgCl_2$ when mixtures of the salts are injected is shown in Fig. 4. LiCl is thirty-two times as effective an antagonizer as Na or KCl (Fig. 5).

Toxic Salt		Antagonizing Salt		
Salt	Concentration	Salt	Effective concentrations	Relative effectiveness of antagonism
$MgCl_2$	$\frac{M}{26}$	LiCl	$2M-\frac{M}{128}$	32
$MgCl_2$	$\frac{M}{26}$	NaCl	$\frac{M}{4}$	1
$MgCl_2$	$\frac{M}{26}$	KCl	$M-\frac{M}{4}$	1

FIG. 5. Summary table of antagonism of LiCl, NaCl, and KCl to toxic concentrations of $MgCl_2$ in its action on the internal protoplasm (injection experiment).

Fig. 6 shows the action of $CaCl_2$ in antagonizing toxic concentrations of LiCl, NaCl, and KCl when mixtures are injected. KCl can be antagonized by $CaCl_2$ 64 times and NaCl 16 times as effectively as can LiCl (Fig. 8). A study of the antagonism by $MgCl_2$ of the action of LiCl, NaCl, and KCl on the internal protoplasm (Fig. 7) demonstrates that KCl can be antagonized by $MgCl_2$ 16,000 times and NaCl 4000 times as easily as can LiCl (Fig. 8).

A comparison of the antagonizing power of $CaCl_2$ and $MgCl_2$ shows that $CaCl_2$ antagonizes LiCl twice as effectively as does $MgCl_2$; $MgCl_2$ is about 126 times more effective than $CaCl_2$ in antagonizing NaCl and about 113 times in antagonizing KCl (Fig. 8).

Combined Immersion and Injection Experiments.

To determine whether the toxic effect of injected LiCl or NaCl could be antagonized if, at the same time, the amebæ were immersed in $CaCl_2$ and in mixtures of $CaCl_2$ with LiCl or NaCl, the following

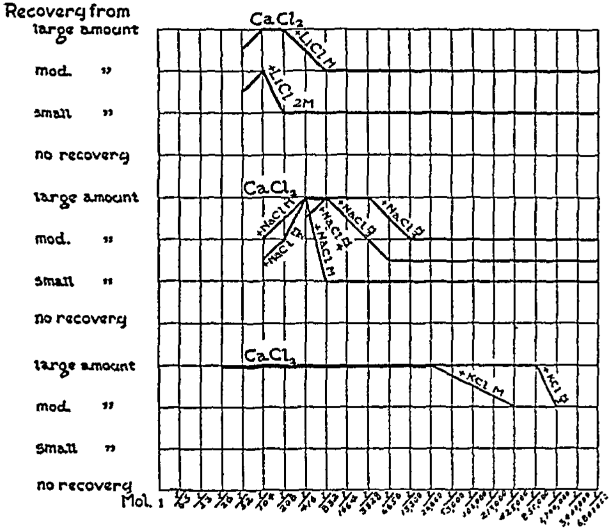

FIG. 6. Antagonism of $CaCl_2$ to toxic concentrations of LiCl, NaCl, and KCl in their action on the internal protoplasm (injection experiment). Abscissæ represent decreasing concentrations of antagonizing salt ($CaCl_2$). Each curve represents a toxic concentration of LiCl, NaCl, or KCl.

experiments were performed: Amebæ were immersed in the following solutions, selected because of their optimum values in immersion tests (*cf.* Fig. 1): M/80 $CaCl_2$, M/32 NaCl and M/320 $CaCl_2$, M/64 NaCl and M/80 $CaCl_2$, M/16 LiCl and M/160 $CaCl_2$, M/32 LiCl and M/80 $CaCl_2$. The amebæ were then injected with M/1 NaCl or 2 M

LiCl, depending upon which of these two salts was in the environing medium. In no case was there any indication that $CaCl_2$ (*cf.* Fig. 6) penetrated the cell from the immersing fluid in sufficient quantity to antagonize the toxic action of the injected LiCl or NaCl.

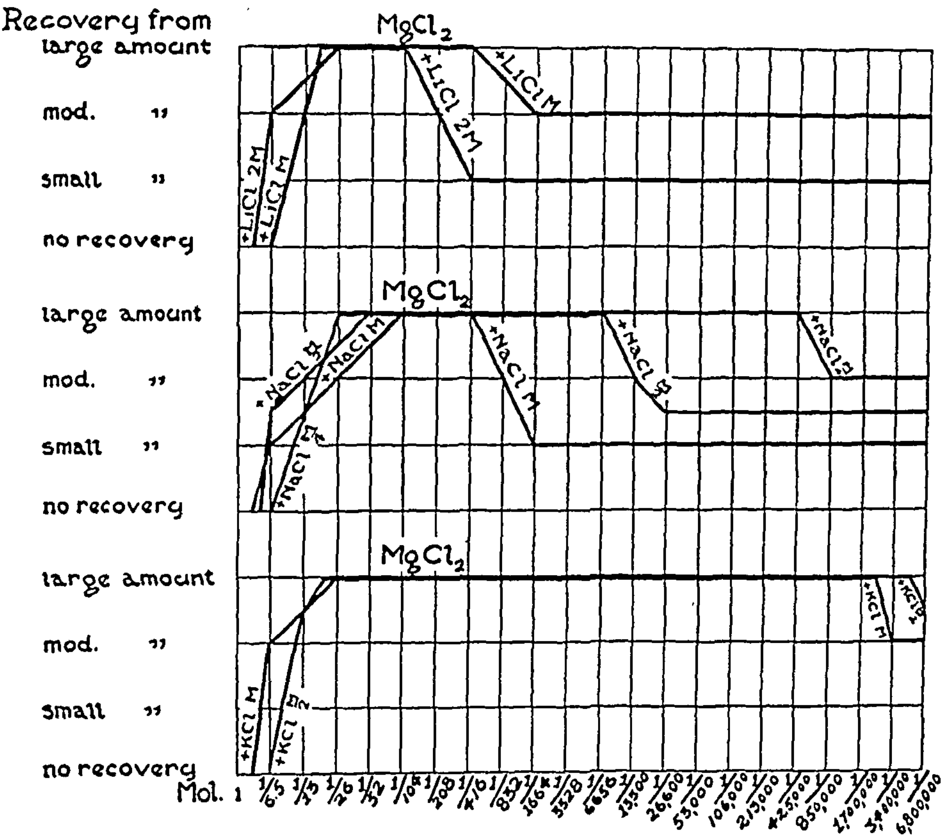

FIG. 7. Antagonism of $MgCl_2$ to toxic concentrations of LiCl, NaCl, and KCl in their action on the internal protoplasm (injection experiment). Abscissæ represent decreasing concentrations of antagonizing salt ($MgCl_2$). Each curve represents a toxic concentration of LiCl, NaCl, or KCl.

One further point merits mention. Although external $CaCl_2$ was unable to prevent the ultimate toxic action of the injected salts, the dispersive effect of these salts on the plasmalemma from the inside tended to be antagonized by the $CaCl_2$ in the medium. This could be seen from the repeated attempts of the disrupted plasmalemma to reform.

Toxic Salt		Antagonizing Salt $CaCl_2$	
Salt	Equivalent toxic concentrations	Effective concentrations	Relative effectiveness of antagonism
LiCl	$\frac{M}{1}$	$\frac{M}{104}$ — $\frac{M}{208}$	1
NaCl	$\frac{M}{4}$	$\frac{M}{416}$ — $\frac{M}{3308}$	16
KCl	$\frac{M}{1}$	$\frac{M}{104}$ — $\frac{M}{26{,}500}$	64

Toxic Salt		Antagonizing Salt $MgCl_2$	
Salt	Equivalent toxic concentrations	Effective concentrations	Relative effectiveness of antagonism
LiCl	$\frac{M}{1}$	$\frac{M}{26}$ — $\frac{M}{104}$	1
NaCl	$\frac{M}{4}$	$\frac{M}{26}$ — $\frac{M}{416{,}000}$	4000
KCl	$\frac{M}{1}$	$\frac{M}{26}$ — $\frac{M}{3{,}000{,}000}$	16,000

FIG. 8. Summary table of antagonism of $CaCl_2$ and $MgCl_2$ to toxic concentrations of LiCl, NaCl, and KCl in their action on the internal protoplasm (injection experiment).

DISCUSSION.

The differences in concentration of Na, K, Ca, and Mg in the cell and in the environment (3), reported by many investigators, is significant in a consideration of the antagonistic action of the salts on protoplasm. Thus in the experiments described in this paper it is to be noted that both $CaCl_2$ and $MgCl_2$ antagonize NaCl in its action on the plasmalemma much better than they do KCl, and that they antagonize KCl in its effect on the internal protoplasm much better than they do NaCl. Incidentally, $MgCl_2$ rather than $CaCl_2$ seems

to act as the effective antagonist of Na in the immersion experiments and of K in the injection experiments.

A second interesting feature of these experiments is the apparent lack of relationship between the degree of antagonizing power and the solidifying or dispersing effect of each salt individually on protoplasm. Thus, although $CaCl_2$ is a better solidifier of the protoplasm than $MgCl_2$, and LiCl has more dispersive effect than NaCl, which, in turn, is more dispersive than KCl, the quantitative measure of antagonizing power of these salts is not directly dependent on these properties alone.

Most of the work reported previously has dealt with the relation of antagonistic salts to changes in permeability or to the maintenance of the normal physical state of protoplasm. Loeb (4–9) demonstrated that the permeability of the membrane of the *Fundulus* egg is involved primarily in antagonistic salt action. Others (10–12) have added evidence regarding the importance of normal permeability as an essential condition brought about by optimum salt relationship. Hansteen-Cranner (13) studied the question of antagonism on plant cells and contributed information on the action of antagonistic salts in maintaining the normal physical state of protoplasm. Lillie (14) studied this question with the jelly of marine eggs, and Spaeth (15) and Clowes (16) discussed the problem from the standpoint of the reversal of phases in colloidal systems. Recently Hirschfelder and Serles (17) considered the action of Mg and Ca on the effect of analgesic drugs in a similar manner. Osterhout (18, 19) believes that antagonism depends upon a shift in chemical equilibrium with resulting changes in physical state and permeability. Van Oijen (20) states that variations in three factors explain antagonism: (*a*) adsorption, (*b*) rate of diffusion into a cell, (*c*) effect on interior of cell. The present study shows directly that to understand the antagonistic relationships of salts it is necessary to consider the particular part of the cell involved and the specific salts used.

CONCLUSIONS.

I. *The Plasmalemma.*

1. On the plasmalemma of amebæ $CaCl_2$ antagonizes the toxic action of LiCl better than it does NaCl, and still better than it does KCl.

$MgCl_2$ antagonizes the toxic action of NaCl better than it does LiCl and still better than it does KCl.

2. $CaCl_2$ antagonizes the toxic action of LiCl and of KCl better than does $MgCl_2$: $MgCl_2$ antagonizes NaCl better than does $CaCl_2$.

II. *The Internal Protoplasm.*

3. The antagonizing efficiency of $CaCl_2$ and of $MgCl_2$ are highest against the toxic action of KCl on the internal protoplasm, less against that of NaCl, and least against that of LiCl.

4. $CaCl_2$ antagonizes the toxic action of LiCl better than does $MgCl_2$: $MgCl_2$ antagonizes the toxic action of NaCl and of KCl better than does $CaCl_2$.

5. LiCl antagonizes the toxic action of $MgCl_2$ on the internal protoplasm more effectively than do NaCl or KCl, which have an equal antagonizing effect on the $MgCl_2$ action.

III. *The Nature of Antagonism.*

6. When the concentration of an antagonizing salt is increased to a toxic value, it acts synergistically with a toxic salt.

7. No case was found in which a potentially antagonistic salt abolishes the toxic action of a salt unless it is present at the site (surface or interior) of toxic action.

8. Antagonistic actions of the salts used in these experiments are of differing effectiveness on the internal protoplasm and on the surface membrane.

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THE PERMEABILITY OF THIN DRY COLLODION MEMBRANES.

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The rate of penetration of water into the sea urchin egg was calculated (1) from the data of Lucke and McCutcheon to be about 1×10^{-5} cc. per hour per sq. cm. membrane per mm. Hg pressure. This figure is of the same order of magnitude as that found for permeable collodion membranes, but since the egg membrane is probably at most only a thousandth as thick as the collodion it is evident that, for the same thickness of membrane, the egg membrane is very much less permeable to water than the collodion. It seemed possible therefore that if a collodion membrane could be made having per unit thickness approximately the same permeability to water as the egg membrane that it would also have the semipermeability characteristic of the egg membrane. Collodion membranes prepared in the ordinary way by placing in water before drying completely are very much more permeable than the egg membranes, whereas the same membranes when completely dried become practically impermeable. Many attempts have been made in this laboratory to obtain a membrane having an intermediate permeability by changing the length of time of drying etc., but it has always been found that there is a sudden transition from a membrane permeable to most electrolytes to one that was impermeable to water. It occurred to the writer that the desired permeability might be secured by making very thin membranes. This was done by pouring U.S.P. collodion into small test-tubes, inverting the test-tubes at once and allowing them to dry in this position for 48 hours. In most cases the membrane pulls away from the glass at the end of this time and may be easily removed. Such membranes were found to be about 2 to 3μ thick, and were strong enough to work

with. Their permeability was tested by filling with 2 M NaCl and immersing in 1 M $AgNO_3$. Membranes showing any precipitate of silver chloride were discarded. In most cases the precipitate could be seen to be due to the presence of holes in the membrane, but in the case of some membranes thin enough to show diffraction fringes the salt appeared to diffuse through.

The permeability for various solutions was then determined by placing 1 cc. of the solution in the membrane fastened to the end of a glass tube and suspending the membrane in 25 cc. of water. The amount of solute which had diffused through was determined by analysis after varying lengths of time, usually 24 hours. In the case of water the increase in volume of the solution was noted, or, for the water vapor determination, the loss in weight of a membrane containing a small amount of water suspended in a desiccator. The permeability to gases was determined by measuring the rate of flow of the gas through the dry membrane under a measured pressure. The general equation for the passage of a substance through such a system may be written

$$\frac{dv}{dt} = \frac{CSP}{h},$$

where v is the amount of substance diffusing, S is the surface, h the thickness, and P the pressure or concentration difference on the two sides of the membrane. The equation would have different forms depending on the mechanism assumed for the passage through the membrane, but for comparative purposes may be used in the general form. If the measurements are made before more than a small per cent of the substance has passed through the membrane, P may be considered constant and the equation used in the differential form, *i.e.*

$$\frac{C}{h} = \frac{\Delta v}{\Delta t S P_0}.$$

In the present experiments v has been expressed in moles, t in hours, and S in sq. cm. P has been used as the vapor pressure in atmospheres in the case of gases or as the diffusion pressure in atmospheres in the case of dissolved substances. (This has been assumed proportional to the concentration and to be equal to 22.4 atmospheres per mole per

TABLE I.

Rate of Diffusion of Substances Through Collodion Membranes.

Substance	H_2O		HCl		NH_3		CO_2
System measured and concentration etc.	H_2O | M/1 NaCl	H_2O vapor | dry air	H_2O | M/1 HCl	HCl gas | air	H_2O | NH_4OH .1M	air | NH_3	CO_2 | air
Moles hr. $^{-1}$ at. $^{-1}$ sq. cm.$^{-1}$ $\times 10^8$	100 (O.P.) 3×10^6(V.P.)	20×10^6(V.P.)	<1 (D.P.)	10,000 (V.P.)	>40 (D.P.)	10,000 (V.P.)	300

Substance	H_2	H_2S	O_2	N_2	air	HCOOH	CH_3COOH	$CH_2ClCOOH$
System measured and concentration etc.	H_2 | air	H_2S | air	O_2 | air	N_2 | air	air | air	M/1 sol | H_2O	M/1 sol | H_2O	M/1 sol | H_2O
Moles hr.$^{-1}$ at. $^{-1}$ sq. cm.$^{-1}$ $\times 10^8$	300	300	70	<1	50	6	4	4

Substance	$CHCl_2COOH$	CCl_3COOH	Butyric acid	$(CH_3)_2CO$	HCHO	C_2H_5OH	Br°
System measured and concentration etc.	M/1 sol | H_2O	M/1 sol | H_2O	M/1 sol | H_2O	M/1 sol | H_2O	M/1 sol | H_2O	M/1 sol | H_2O	M/1 in KBr | H_2O
Moles hr. $^{-1}$ at. $^{-1}$ sq. cm. $^{-1}$ $\times 10^8$	1	.4	2	8	2.5	2	3

Substance	I°	Urea	$(NH_4)_2CO_3$	$NaHCO_3$	$HgCl_2$
System measured and concentration etc.	N/10 I° in KI | H_2O	M/1 | H_2O	.1 M sol | H_2O	.1 M sol | H_2O	.1 M sol | H_2O
Moles hr. $^{-1}$ at. $^{-1}$ sq. cm. $^{-1}$ $\times 10^8$	2	about 1	2	1	1

The following substances were tested in M/10 solution, where possible, and found to pass at less than 10^{-8} moles per hour per atmosphere diffusion pressure per sq. cm.: Lactic acid, glycine, oxalic, succinic, sugar, glycerine, HCl, H_2SO_4, HNO_3, HBr, NaCl, KCl, KI, NH_4Cl, $AlCl_3$, $FeCl_3$, $CuCl_2$, $SnCl_4$, $CaCl_2$, Na_2S, NaOH, LiOH, KOH, $Ba(OH)_2$, $Ca(OH)_2$, and the following ammonium salts: -CrO_4, -citrate, -SO_4, -salycilate, -Br, -Cl, -oxalate, -nitrate, -tartrate, -molybdate.

O.P. = per atmosphere osmotic pressure.
V.P. = " " vapor "
D.P. = " " diffusion "

liter.) In the case of water the rate may be expressed either per atmosphere vapor pressure or per atmosphere osmotic pressure. The results therefore are in terms of moles per hour per atmosphere per sq. cm. membrane surface. All experiments were carried on at about 25°C. The figures are the average of 6 to 8 determinations. The variation in the membranes is considerable and the figures are significant only as to the order of magnitude.

The results of the experiments are summarized in Table I in which the substances have been arranged approximately in the order of their permeability. The membrane is permeable to water, ammonia, HCl gas, much less so to CO_2, O_2, and H_2 and to weak acids, whereas it is practically impermeable to salts, strong acids and bases, and sugar and glycerine. The permeability in a general way resembles that found for living cells by Osterhout (2), Lillie (3), and others. The results are qualitatively the same as those of Collander (4) and Fujita (5). The value for water, 1×10^{-6} moles hr.$^{-1}$, sq. cm.$^{-1}$, at.$^{-1}$ may be compared to 3×10^{-4}, which is the approximate figure for the permeability of the sea urchin egg in the same units. Since these membranes are still probably several hundred times thicker than the egg membrane the permeability per unit thickness is probably of about the same order of magnitude. The permeability to NH_4OH, acetone, etc., is not due to an effect on the membrane, since no chloride ion passes when NaCl is present in the solution during the experiment. Alkali stronger than about .01 M destroys the semipermeability, as do concentrated solutions of alcohol, acetone, acetic acid, etc. The results with gases show that they do not pass through holes in the membrane, since the rate of effusion would then be inversely proportional to the square root of the density and hydrogen would pass most rapidly; whereas the experiments show that a number of gases pass much more rapidly than hydrogen. The membrane therefore in the case of gases cannot be considered as a sieve. It is evidently more nearly analogous to a rubber membrane than to a porous plate. The same conclusion is brought out by the fact that immersing the membrane in water has no effect on the rate of passage of the gas. When the membrane is immersed any pores would be filled with water and the rate of passage of the gas would therefore be changed if it flowed through pores in the dry membrane. Taken as a whole, the results

indicate that the substance dissolves in and then diffuses through the membrane. The rate of passage will therefore be determined by the diffusion coefficient of the substance in collodion and by its solubility in collodion, since the latter property fixes the concentration gradient in the membrane. In general, diffusion coefficients differ much less for various substances than solubilities so that the solubility is probably the more important factor.

SUMMARY.

Dry thin collodion membranes have been prepared which are permeable to water, ammonia, weak acids of low molecular weight, HCl gas, O_2, CO_2, and H_2S, but are impermeable to strong electrolytes and substances of high molecular weight. The permeability to gases does not depend on the density, so that the gases do not pass through pores in the membrane.

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THE EFFECT OF RENNIN UPON CASEIN.

II. Further Consideration of the Properties of Paracasein.

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I.

INTRODUCTORY.

In a preceding investigation (1) upon certain properties of paracasein, we attempted to identify paracasein by physicochemical means.

Paracaseins were prepared by coagulating milk by means of rennin or pepsin preparations, purifying the resulting paracasein in the same way as was done in the case of casein (2). Such preparations, free from any proteolytic enzyme, were then tested for their solubility in water and for their solubility in dilute NaOH solutions at two temperatures. It was found that although paracasein at about 6°C. dissolves in water to the same extent as casein, the capacity to bind base was distinctly different from that of casein. While casein at 21 to 37°C. dissolves in combination with base to the extent of about 2100 gm. per mol of NaOH added (2, 3), most of the paracasein preparations combined with NaOH in this temperature range with a combining weight of 1450 gm.

The ratio of the equivalent combining weight of casein to the combining weight of paracasein is as 1 to 1.45. Solubility measurements at 5°C. indicated that the same relationship held true for these proteins at low temperature.

In the present investigation, we have studied further some of the other properties of paracasein preparations, among which are the hydrogen ion activity in systems composed of paracasein and base, and the maximum base-binding capacity of certain paracaseins.

In a previous investigation, we have reported solubility measurements upon Paracasein Preparation VI. Unlike the other five paracasein preparations this one had a combining weight of about 1700 gm. with NaOH. Some of the properties of this paracasein have been investigated further.

During the course of the preparation of paracasein we have noted (1) the appearance of certain hydrolytic products. This suggested the possibility of preparing paracasein by a partial hydrolysis of casein in alkaline solutions.

Finally, we shall attempt to summarize the differences between paracasein and casein.

II.

The Hydrogen Ion Activity in a System Composed of Paracasein and NaOH.

The estimate of the hydrogen ion activity in systems composed of paracasein and NaOH was made both colorimetrically and electrometrically.

Colorimetrical Method.— A sample of paracasein, previously washed with distilled water, was placed in a bottle. This suspension was vigorously agitated by means of a stirrer and NaOH was added, then a 10 cc. sample was pipetted out, filtered, and the pH of the solution determined colorimetrically, using methyl red or brom thymol blue. An additional 10 cc. of NaOH was then added to the bottle. Thus the volume of the suspension was always kept constant.

Let m be the initial amount of paracasein in the system. Let a be the total volume, q the volume of the sample taken for each measurement. The amount of paracasein left after the nth removal is given by the following expression:

$$x = \text{gm. of paracasein left} = m(1-q/a)^n$$

$q = 10$ cc., $a = 200$ cc. and $m = 3.47$ gm. The equation assumes the form

$$\log x = 0.5403 - 0.023n.$$

From this equation and known values of n, the values given in Table I were calculated.

Electrometrical Method.—To known amounts of paracasein preparations were added varied amounts of NaOH. The samples were then shaken for a time extending from 9 to 48 hours at temperatures ranging from 20 to 25°C. The hydrogen ion activity was determined potentiometrically against a saturated calomel electrode. Saturated KCl was used for a bridge. The data were not corrected for the diffusion potential.

TABLE I.

Titration of Paracasein.

Colorimetric Titration.

Initial amount of protein: 3.47 gm. in 200 cc.

n (1)	Mols NaOH $\times 10^{-5}$ to 1 gm. (2)	pH (3)
	Paracasein II	
4	5.34	5.4
5	9.40	(5.8)
6	13.9	5.85
7	18.8	5.9
8	24.3	6.0
9	32.0	6.05
10	36.8	6.2

Electrometric Titration.

Mols NaOH $\times 10^{-5}$ to 1 gm.	Temperature	pH
	Paracasein I	
	°C.	
21.3	23.9	6.12
37.7	24.1	6.36
52.2	24 6	6.65
69.5	22.6	7.16
	Paracasein II	
20.5	23.1	5.98
65.0	21 0	6 77
70.0	23.6	7.15

The results of the electrometrical titration of Paracasein Preparations I and II are given in Table I. Both the colorimetric titrations

of Paracasein Preparation II and the electrometric titration of Paracasein Preparations I and II are graphically represented in Fig. 1, together with the titration curve of casein (4).

It is evident that paracasein behaves as though it were a stronger acid than casein. At any point of the titration curve an equal amount of base brings casein to a less acid reaction than paracasein.

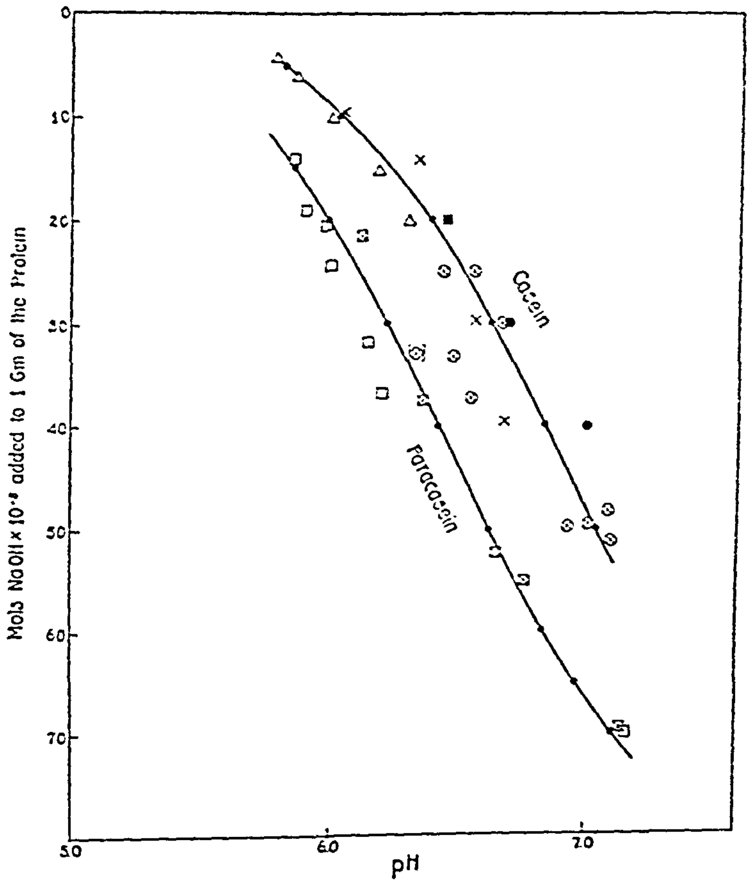

FIG. 1. The titration of paracasein.

By comparing the combining weights of casein and paracasein toward NaOH we have already arrived (1) at the conclusion that paracasein has a greater number of acid groups than casein. A comparison of the titration curves of these two proteins substantiates this conclusion: paracasein is a stronger acid than casein.

III.

The Maximum Base-Combining Capacity of Paracasein.

The maximum base-combining capacity of a protein may be defined as being the largest amount of base which will combine with a given amount of protein.

The amount of base bound may best be estimated (5) by calculating the amount of free base in solution and subtracting this value from the amount of base added. The difference yields the amount of base bound by the protein. When this difference remains constant, upon further addition of base, the maximum base-combining capacity has been reached.

The maximum base-combining capacity of paracasein was estimated as follows: To a known amount of the protein an excess of base was added, the solution shaken for a short period of time, and its hydrogen ion activity estimated electrometrically. The base found was calculated following the method already described (5). The results of these experiments are given in Table II.

In an extensive investigation upon the maximum base-combining capacity of casein, Cohn and Berggren (5) found that this property of casein depends upon the method used for the preparation of the protein. Preparations which were brought to an alkaline reaction during their purification were found to bind more base than those which were never subjected to alkalinities greater than pH 7.00. Two limits were thus discovered, the lowest representing a casein of a maximum base-combining capacity of 138×10^{-5} mols of NaOH per 1 gm., and the highest, the casein modified by an alkaline treatment, binding as much as 185×10^{-5} mols of NaOH per 1 gm. These two limits are of considerable interest to the chemistry of casein. As it has been shown elsewhere (3) these limits yield multiple relationships with other constants derived from the study of casein.

The reciprocal of the equivalent combining weight of paracasein, 1450 gm., *i.e.*, the amount of base necessary to dissolve 1 gm. of paracasein, is 69×10^{-5} mols of NaOH. The corresponding figure for casein is 47.5×10^{-5} mols (2). The difference between these two values is 21.5×10^{-5} mols.

TABLE II.

The Maximum Base-Combining Capacity of Paracasein.

Temperature 22.0 ± 0.5°C.

Preparation No. (1)	Experiment No. (2)	Paracasein in 1 liter (3)	Concentration of NaOH (4)	pOH (5)	pNaOH (pOH − pγ) (6)	Concentration of free NaOH (7)	Base bound (4) − (7) (8)	Base bound 1 gm. paracasein (8)/(3) (9)
		gm.	*mols*			*mols*	*mols*	
I	1	12.70	0.030	2.132	2.073	0.0085	0.0215	0.0169
I	1	12.70	0.030	2.148	2.089	0.0082	0.0218	0.0172
I	1	12.70	0.030	2.143	2.082	0.0082	0.0218	0.0172
I	2	13.02	0.040	1.808	1.743	0.0181	0.0219	0.0168
I	2	13.02	0.040	1.817	1.752	0.0177	0.0223	0.0171
I	2	13.02	0.040	1.811	1.746	0.0179	0.0221	0.0170
I	3	13.34	0.050	1.616	1.545	0.0285	0.0215	0.0161
I	3	13.34	0.050	1.616	1.545	0.0285	0.0215	0.0161
I	3	13.34	0.050	1.631	1.560	0.0275	0.0225	0.0169
Average								0.0168
II	1	12.40	0.030	2.190	2.131	0.0074	0.0226	0.0182
II	1	12.40	0.030	2.183	2.124	0.0075	0.0225	0.0181
II	1	12.40	0.030	2.199	2.140	0.0073	0.0227	0.0183
II	2	11.60	0.040	1.812	1.746	0.0179	0.0221	0.0190
II	2	11.60	0.040	1.795	1.730	0.0186	0.0214	0.0184
II	2	11.60	0.040	1.795	1.730	0.0186	0.0214	0.0184
II	3	11.86	0.050	1.593	1.522	0.0301	0.0199	0.0168
II	3	11.86	0.050	1.608	1.537	0.0290	0.0210	0.0177
II	3	11.86	0.050	1.610	1.539	0.0289	0.0211	0.0178
II	3	11.86	0.050	1.599	1.528	0.0296	0.0204	0.0172
Average								0.0180
III	1	7.70	0.030	1.812	1.753	0.0177	0.0123	0.0160
III	1	7.70	0.030	1.835	1.776	0.0168	0.0132	(0.0171)
III	1	7.70	0.030	1.817	1.756	0.0175	0.0125	0.0162
III	1	7.70	0.030	1.823	1.764	0.0172	0.0128	0.0166
III	2	7.72	0.040	1.617	1.552	0.0281	0.0119	0.0154
III	2	7.72	0.040	1.617	1.552	0.0281	0.0110	0.0154
III	2	7.72	0.040	1.619	1.554	0.0279	0.0121	0.0157
III	3	7.76	0.025	1.967	1.912	0.0122	0.0128	0.0167
III	3	7.76	0.025	1.961	1.906	0.0124	0.0126	0.0164
Average								0.0164

The lowest amount of base which paracasein binds at maximum is 164×10^{-5} mols per 1 gm. (Table II). The lowest amount for casein, according to Cohn and Berggren (5) is 136×10^{-5} mols. The difference is 28×10^{-5} mols.

Comparing this value with the one representing the amount of base which paracasein binds in excess over casein in passing into solution, the following conclusion may be drawn: the increase of the maximum combining capacity of paracasein over the combining capacity of an "unmodified" casein is sufficient to account for the excess of base bound by paracasein over casein while passing into solution.

IV.

Is It Possible to Produce Paracasein from Casein by a Partial Hydrolysis of the Latter?

Paracasein binds more base at saturation than does an "unmodified" casein. This suggested the possibility that a casein preparation having a high maximum base-binding capacity, bears a certain relation to paracasein. Therefore, Casein Preparation XXD was selected, the maximum base-binding capacity of which was determined by Cohn and Berggren (5). This preparation, treated during the course of its preparation by an excess of alkali, bound 184×10^{-5} mols of NaOH per gm.

The solubility measurements in NaOH at 5°C. yielded the following figures: With 1.00×10^{-5} mols of base $-$ 1.92 mg. N passed into solution,—with $5.00 \times 10^{-5} - 10.2$ mg. 1330 gm. passed into solution per 1 mol of NaOH added. According to our previous experiments (3) the combining weight of a natural casein in a corresponding solubility range and temperature, is 1300 gm. Casein Preparation XXD thus behaves identically to a natural casein in this respect and bears no relation to paracasein.

During the course of the preparation of paracasein we noted the appearance of hydrolytic products soluble in water at pH. 4.7. This suggested the possibility that paracasein was a product of a partial hydrolysis of casein. We, therefore, sought to produce paracasein from casein by a partial hydrolysis of the latter.

For this purpose, part of the Casein Preparation XXV was dissolved by an addition of NaOH until the pH of the solution reached

6.5. The solution was left undisturbed for 1 month at a temperature of about 24°C. Then the casein in solution was precipitated by a slow addition of HCl to pH 4.7 and extensively washed in distilled water. Samples of this casein were then brought into intimate contact with known amounts of NaOH in the way described (2), and the amount of the nitrogen in solution determined. The results of this experiment are recorded in Table III (Experiment 8).

TABLE III.

The Solubility in NaOH of Casein Preparations Partially Recovered from Hydrolyzed Solutions.

Temperature at which the solubility measurements were carried out: 25°C.

Experiment No. (1)	Casein preparation (2)	Treatment (3)	Time for equilibration (4)	Amount of NaOH added (5)	Solubility mg. N dissolved per 100 gm. of H_2O (6)
			hrs.	*mols* $\times 10^{-5}$	
8	XXV	Dissolved for 30 days at pH 6.5 at 24°C., reprecipitated and washed at isoelectric point	$3\frac{1}{2}$	2.00	5.7
"	"		"	"	5.9
"	"		"	4.00	12.9
"	"		"	"	12.7
18	"	Dissolved for 70 hrs. at pH 11.0 at 30°C., reprecipitated as above	7	2.00	6.0
"	"		"	4.00	13.3
21	"		4	6.00	18.7
18	"		7	"	18.2
21	"		4	8.00	25.2
53	XXVII	Dissolved for 11 days at pH 9.40 at 20°C. 33 per cent hydrolyzed, reprecipitated as above	20	2.00	6.2
"	"		"	6.00	18.8
55	"		"	8.00	23.8

Another part of the same preparation was subjected to a more vigorous hydrolysis. It was brought to pH 11.0 and kept for 70 hours at a temperature of 30°C. The casein left unhydrolized, was precipitated as before, washed, and its solubility in NaOH determined (Experiments 18, 21, Table III).

An attempt not only to hydrolyze part of the casein, but also to measure the amount of casein hydrolyzed, was made upon Casein

Preparation XXVII. Part of this preparation was brought by NaOH to pH 9.40 and kept dissolved for 11 days at a temperature of 20°C. The casein was then precipitated as before and the amount of soluble

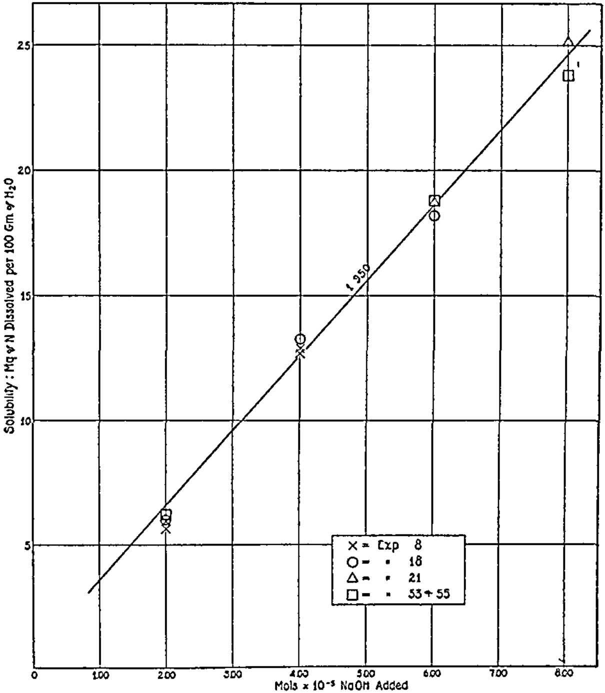

FIG. 2. The solubility of casein in NaOH, partially recovered from hydrolyzed solutions.

nitrogen at pH 4.7 determined. The amount of nitrogen found in solution was reckoned as the amount of casein hydrolyzed. 33 per cent of the original amount of casein was found to be in solution.

The results of these experiments are recorded in Table III (Experiments 53 and 55).

In Fig. 2 we have plotted the results and calculated the value of the equivalent combining weight.

Upon inspection of this chart as well as of Table III it seems evident that all the caseins have identical solubility in NaOH, and furthermore that their capacity to bind base is similar to the natural casein. 2100 ±100 gm. may be assigned (2, 3) to the equivalent combining weight of casein. The result of our calculation yields 1950 gm.

It seems, therefore, plausible to conclude from these experiments that casein recovered from the hydrolysate is practically identical in its solubility in NaOH with the original casein. If an excess of alkali (5) increases the maximum base-combining capacity of casein, this increase is not at the expense of acid groups involved in the passage of this protein into solution. By hydrolysis of the sodium salt of casein, no body bearing a resemblance to paracasein was obtained. It is possible to conclude that the hydrolysis promoted by an excess of NaOH is not identical to the reaction promoted by rennin or pepsin.

We hope, in the future, to extend these observations to the hydrolysis of casein in the presence of $Ca(OH)_2$.

V.

Paracasein Preparation VI.

In the preceding investigation upon the property of paracasein (1) we found that one of the six paracasein preparations studied, namely, Preparation VI, dissolved in NaOH at a rate of 1700 gm. per mol of base added. The combining capacity of this preparation was found to be different from the others, whose combining weights average around 1450 gm. Several explanations may be presented to account for the property of this preparation. It may be supposed that this preparation is an intermediary product of the reaction of rennin upon casein. That is, upon further action of rennin upon this preparation we should expect as a result a modification of casein having the combining weight of 1450 gm. Another explanation may be sought in the

supposition that the preparation is a mixture of natural casein and paracasein. In this case, when a given amount of this preparation is dissolved by NaOH, the combining capacity should not remain a con-

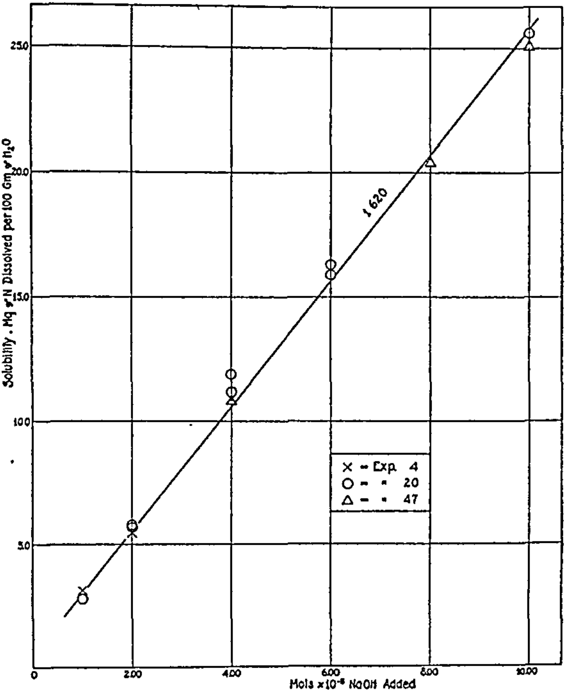

FIG. 3. The solubility of Paracasein VI R in NaOH.

stant, since according to this explanation we have present two chemical individuals, one having the combining capacity of 2100 gm. and the other 1450 gm.

In order to study the effect of rennin upon Paracasein VI, 6 gm. of this preparation were brought to pH 6.8 by the addition of NaOH. 0.01 gm. of Hansen's salt-free rennin preparation was added and the solution kept at 27°C. for 24 hours. The preparation was then precipitated and purified in the same way as has been already described (1). The suspension was then made to contain 30 mg. of nitrogen per 100 cc. and its solubility in NaOH at 25°C. investigated. The results of these experiments are recorded in Table IV and the combining weight calculated from these data in Fig. 3.

TABLE IV.

The Solubility of Paracasein Preparation VI R in NaOH at 25°C.

Amount of paracasein: 30 mg. N.

Experiment No. (1)	Date (2)	Time for equilibration (3)	Amount of NaOH added (4)	Solubility mg. N dissolved per 100 gm. of H_2O (5)
		hrs.	*mols* $\times 10^{-3}$	
22	Aug. 3, 1926	4	1.00	3.1
54	Feb. 16, 1927	20	"	2.8
22	Aug. 3, 1926	4	2.00	5.5
54	Feb. 16, 1927	20	"	5.7
57	Mar. 14 "	"	"	5.8
22	Aug. 3, 1926	4	4.00	10.9
54	Feb. 16, 1927	20	"	11.2
57	Mar. 14 "	"	"	11.9
56	Feb. 16 "	47	"	10.8
"	Feb. 19 "	20	6.00	15.9
57	Mar. 14 "	"	"	16.3
56	Feb. 19 "	47	8.00	20.4
57	Mar. 14 "	20	10.00	25.6
56	Feb. 19 "	47	"	25.1

The following conclusions may be drawn from these experiments. First, the combining capacity of this preparation did not change appreciably after treatment by rennin, and furthermore every fraction of it (Fig. 3) is endowed with practically the same combining capacity with NaOH.

This preparation did not show any sign of modification after being kept for a long time at the isoelectric point at 5°C. Solubility experiments carried after an elapse of 7 months (Table IV) indicated the same solubility. Similarly, the solubility of Paracasein II was found to be unaffected by time. Therefore, it is plausible to conclude that paracaseins are relatively stable bodies.

The result of this investigation indicates that it is probable that Paracasein VI is not a mixture of casein and paracasein, and is not appreciably modified in the form of a sodium salt by a further treatment by rennin.

VI.

Is Casein Stable in Regard to Its Solubility in NaOH?

The preparation of paracasein takes on the whole 1 week more than the preparation of casein. If casein is unstable, it may be supposed that paracasein is nothing more than a denatured casein, denatured by being kept longer, but not denatured by rennin or pepsin.

Our experience with the solubility of casein in NaOH indicates that casein is a fairly stable body, keeping its qualities a good many weeks after its preparation. This question, however, is of such importance, that we decided to check up once more our evidence concerning the stability of casein. For this purpose Casein Preparation XXV, available in sufficient quantity, was selected. It was prepared in October, 1925, from non-pasteurized milk, by the method already described in detail (2). From that time on it was kept under toluene at about 5°C. In July, 1926, the preparation was dissolved to pH 6.3 and reprecipitated, washed Cl-free, and used in part for some solubility measurements.

In June, 1927, the preparation was washed six times with distilled water and the experiment reported in Table V was undertaken. The results of this experiment indicate that Casein XXV after 1 year and 7 months still possessed a combining weight of about 2100 gm.

The conclusion may be drawn that casein is relatively stable in respect to its capacity to bind NaOH. Paracasein, thus, in this case, cannot be regarded as a product of denaturation of casein without the interference of rennin or pepsin.

TABLE V.

The Solubility in NaOH of Casein Preparation XXV, Kept for 1 Year and 7 Months at 5°C.

Temperature 25°C.

Experiment 70.

Time for equilibration: 20 hours.

Amount of protein: 50 mg. N.

NaOH added (1)	Mg. N dissolved (2)	Equivalent weight (by difference) (3)
mols $\times 10^{-4}$		
2.00	5.25	
6.00	17.7	2000
10.00	31.2	2150
Average		2075

VII.

SUMMARY AND CONCLUSIONS.

The properties of the paracasein and casein preparations studied are compared in Table VI.

TABLE VI.

The Comparison of the Properties of Casein and Paracasein.

Property (1)	Temperature (2)	Casein (3)	Paracasein (4)
	°C.		
Solubility in NaOH: gm. protein bound by 1 mol	25.0	2100 (2)	1450 (1) (1650)
Same. Gm. protein dissolved by 1 mol (with small amounts of NaOH)	5.0	1300 (3)	810 (1)
Solubility in water in uncombined state: mg. N per liter	6±1	7.0 (3)	6.8 (1)
Titration curve in the solubility range	22±2	Weaker acid than paracasein	Stronger acid than casein
Maximum base-binding capacity, mols NaOH per 1 gm.	21±2	From 136 to 166 $\times 10^{-5}$ mols NaOH per 1 gm. (5)	From 164 to 180 $\times 10^{-5}$ mols NaOH per 1 gm.

Certain other problems dwelt upon in this investigation may be summarized as follows:

I. Casein retains its characteristic solubility in NaOH: (1) after being exposed to a high degree of alkalinity during its preparation, (2) when recovered from partially hydrolyzed solutions in NaOH, and (3) after being kept for a prolonged time at the isoelectric point at 5°C.

II. It follows from I, that: (1) paracasein is not identical to casein modified by an excess of alkali, and that (2) this protein was not produced from casein by a partial hydrolysis of the latter in presence of NaOH.

I am indebted to Dr. E. J. Cohn for helpful advice and encouragement throughout the research upon paracasein. I am also indebted to Miss R. E. L. Berggren for technical assistance.

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THE RELATION BETWEEN VISUAL ACUITY AND ILLUMINATION.*

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I.

Visual Acuity and Intensity.

1. Data.—It is common experience that the fineness of detail which the eye can distinguish varies with the intensity of illumination. A measure of the capacity for distinguishing details is visual acuity. This is defined as the reciprocal of the angular distance which must separate two contours in order that they may be recognized as discrete. The relation between visual acuity and illumination was first investigated in 1754 by the astronomer Tobias Mayer. He believed to have found that visual acuity increases as the sixth root of the intensity of illumination.

Since Mayer many investigators have tried to find the exact nature of the relationship between visual acuity and illumination. Uhthoff (1886), who reviewed the data 40 years ago, records about twenty contributions to this particular phase of vision. Most of these data cover only a very small range of illuminations, and therefore merely confirm daily experience. Nevertheless, even a small range enabled Posch (1876) to conclude that visual acuity varies very nearly as the logarithm of the illumination intensity. This relation is borne out by the more comprehensive data of Klein (1873) and Cohn (1883), though neither of these authors drew such a conclusion.

Uhthoff (1886; 1890) himself investigated the matter further by using white and colored lights covering a great range of illuminations.

* A preliminary account of these results was presented to the XIIth International Physiological Congress at Stockholm, August 3 to 6, 1926. *Cf. Skand. Arch. Physiol.*, 1926, xlix, 146.

From his data it is apparent that in a general way visual acuity varies in proportion to log I; the relation between the two is not strictly rectilinear, but sigmoid. His data are excellent, and would suffice

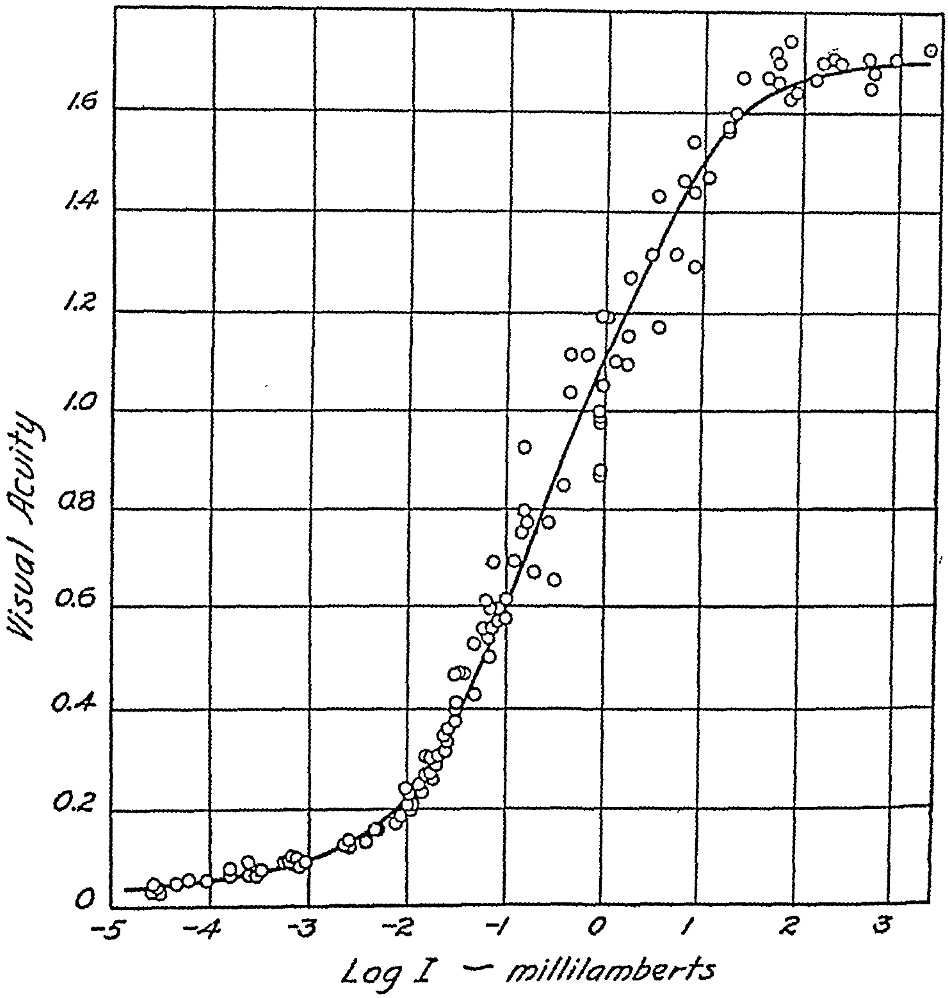

FIG. 1. Relation between visual acuity and illumination. Data of Koenig (1897). The original intensities have been multiplied by 0.072 to convert them into millilamberts. The curve is a theoretical one derived as explained in the text.

for an accurate statement of the relationship, were it not for the fact that a few years later Koenig (1897) redetermined the influence of illumination on visual acuity in such comprehensive detail that his

data have become classic. The measurements for white light are reproduced in Fig. 1. Koenig drew three straight lines through his data: one line with a small slope through the low points; another line with a steep slope through most of the points; and finally a horizontal line at very high intensities, representing a condition, known since Tobias Mayer, in which visual acuity no longer increases with illumination. For each of these straight lines Koenig wrote that visual acuity varies directly as the logarithm of the illumination.

Since Uhthoff's and Koenig's experiments the number of people who have measured this relationship is not large. Up to 1912 they are recorded in an excellent memoir by Löhner (1912); an earlier review is by Zoth (1905). After that, with the recent exception of Roelofs and Zeeman (1919) there have been no significant contributions to this subject. All combined, the investigations since Koenig (*e.g.* Rice, 1912 and Ferree and Rand, 1923) have not covered the range of illumination necessary for a complete statement of this function of the eye. None the less, scattered as they are, the data indicate clearly that the relationship between visual acuity and illumination is of the form found by Koenig.

Since it is proposed to use Koenig's data as a basis for a study of this relationship, it may be well to give for comparison an example of the more recent data which cover the largest range of illuminations. Roelofs and Zeeman measured the visual acuity of their own eyes, using four different types of test objects covering only a part of the visual range with each. Their measurements are in terms of the distance which the test object (parallel lines, small squares, etc.) must be in order that it may be recognized as discontinuous. It has been necessary to convert these distances into units of visual acuity comparable to those used by Koenig, which are practically the common ones of ophthalmological practice (Koenig (1897)).[1] In order to bring the results of the four separate series of Roelofs and Zeeman together it has been necessary to multiply each by a factor. The data recalculated in this way are given in Fig. 2. It is only necessary to compare Fig. 2 with Fig. 1 to realize that they are an excellent corroboration of Koenig's work; and that Koenig's data are still the best and most com-

[1] Koenig (1897), p. 379.

plete statement of the manner in which visual acuity varies with the intensity of the illumination.

2. *Statement of Problem.*—The phenomenon that visual acuity varies in a definite way with illumination has been known for about 150

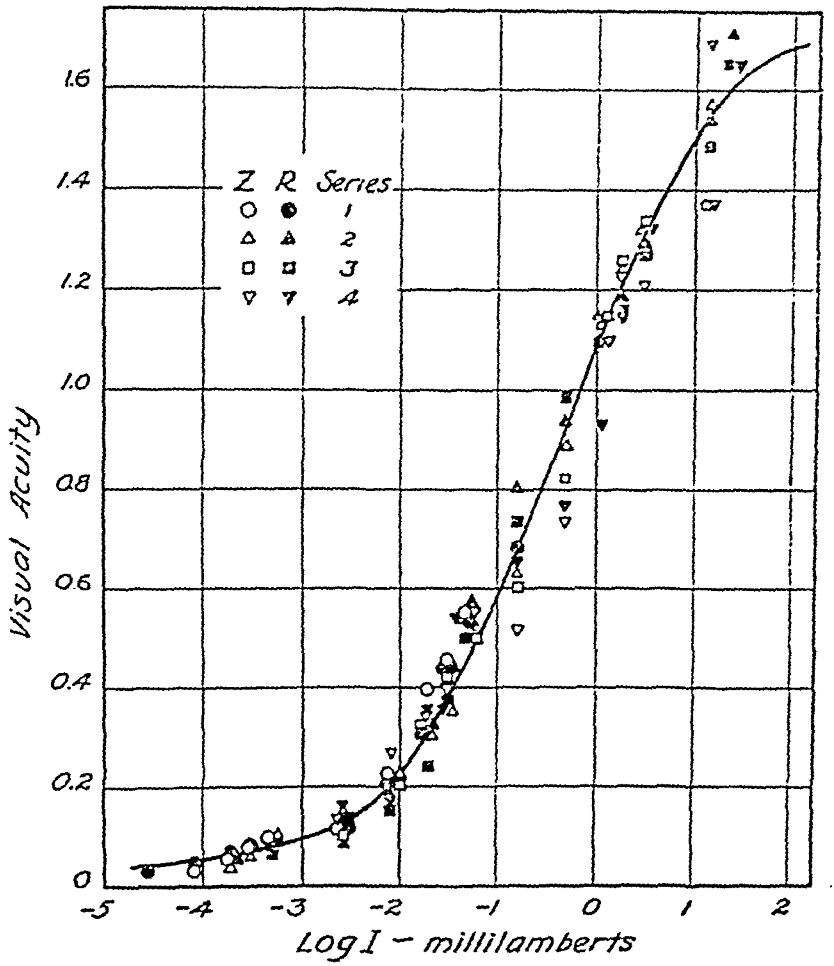

FIG. 2. Relation between visual acuity and illumination. Data of Roelofs and Zeeman (1919). The intensities have been multiplied by 0.18 to convert them into millilamberts. The measurements in Series 1 were made with black stripes; in Series 2 with large squares; in Series 3 with small squares; and in Series 4 with parallel lines. To bring them into conformity with each other and with Koenig's units of visual acuity as given in Fig. 1, it has been necessary to multiply the distances as given in Series 1 by 0.000823; in Series 2 by 0.00201; and in Series 3 and 4 by 0.00329.

years. An exact description of the relationship has been in existence for about 30 years. Nevertheless, an extensive search through the literature of vision has revealed only two occasions on which this relation has elicited any critical comment.

Helmholtz (1896) made the astute suggestion that poor visual acuity at low intensities might be related to the coarseness of intensity discrimination under similar conditions. Later, Broca (1901) supposed that a part of the increase of visual acuity with illumination could be accounted for on the assumption that at high illuminations the retinal pigment migrates between the cones, compresses them and thus decreases their diameter. For the decreased visual acuity at low illuminations Broca further supposed that the connection between retinal elements and nerve fibers is not fixed, but that the number of elements which communicate with a single fiber increases as the intensity decreases. Broca failed to realize that decreasing the diameter of the cones does not alter their average distance apart. Moreover, there is no evidence for retinal pigment migration in man (Arey, 1915).

The only real contribution to an understanding of the data in Fig. 1 was made by Koenig himself on the basis of the duplicity theory. Koenig interpreted the lower points, which he connected with a straight line of small slope, as the expression of *the function of the rods in the* periphery; the rest of the points, connected by a line of large slope, as the expression of the activity of the cones in the fovea. This can be verified by anyone who makes the observations. At low illuminations fixation is peripheral; at high, it is foveal.

This division of the data into rod visual acuity and cone visual acuity is positive contribution, and on it rests the present paper. However, there still remains the central, all important fact that visual acuity, as mediated either by rods or by cones, varies in a very definite manner with the illumination. It is my purpose here to propose an explanation of this relationship, and to show how it accounts for the data in quantitative detail.

II.

Nature of Explanation.

1. Retinal Basis of Visual Acuity.—The fineness of detail which a surface can register depends upon the number of receiving elements

present in a unit area of the surface. In other words, its resolving power varies in inverse proportion to the average distance between the centers of the sensitive elements. This is very evident in such a case as the photographic plate.

The retina is a surface of this kind since it is composed of discrete rods and cones which function as individual units or as groups of units. The way in which visual acuity varies with illumination indicates the way in which the resolving power of the retina varies. A low visual acuity means that the average distance between the retinal elements is large; whereas a high visual acuity means that the distance is relatively small. To account for the large variation in visual acuity with illumination, one must suppose that the number of sensitive elements per unit area of retina can and does vary nearly a hundredfold. But the number of rods and cones in the retina is fixed anatomically. Therefore it is necessary to assume that the number of these elements is variable functionally.

2. *Assumptions.*—To accomplish this, I have made the almost obvious supposition that in a given retinal area, the sensitivity of the individual rods or cones is not uniform, but is distributed in relation to the intensity of illumination in the manner of populations, errors, and the like, familiar from the work of statisticians. Fig. 3 gives the sensibility distribution which has to be assumed for the cones in the fovea and for the rods in the periphery in order to describe the data of Figs. 1 and 2. The curves are absolute in their abscissa values, but are relative in their ordinate values, since these depend on the size of the unit area concerned. The rod and cone curves are identical in shape, but are different in position and in relative height of ordinates.

These sensibility distribution curves of Fig. 3 may be considered as differential curves. They represent the relative number of rods and cones whose threshold is to be found at a given retinal illumination. As such the curves show that the distribution is typically skew. What we wish to know, however, is not merely the number of elements whose threshold lies at a specific illumination, but the total number of elements which are functional at a given illumination. This is given by the integral form of the curves in Fig. 3, and corresponds at any value of I to the area under the curve to the left of that value. The integral forms of these two curves are given in Fig. 4.

Visual acuity measures the resolving power of the retina; and since this depends on the number of elements functioning in a given area, it follows that visual acuity varies directly with the number of functional rods or cones in a unit area of illuminated retina. Since the size of this "unit area" is not known, the ordinates in Fig. 4 are arbitrarily chosen to read directly in units of visual acuity. Conversely

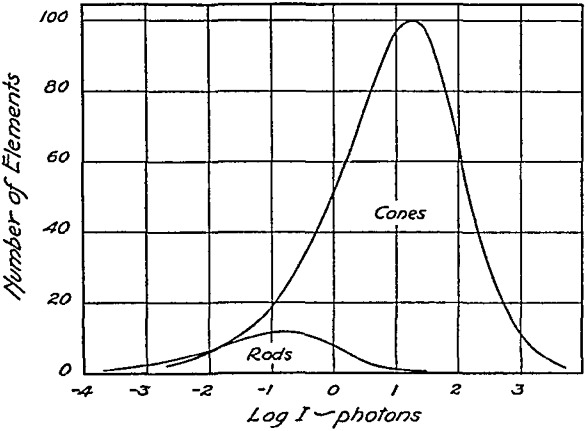

FIG. 3. Distribution of thresholds of rods and cones. The intensities are in photons, a unit introduced by Troland to represent the retinal illumination produced when the eye looks at a brightness of 1 millilambert through a pupil of 1 sq. mm. The two curves are identical in form, but different in position and in size of ordinates.

this will furnish a method of finding what the dimensions of a unit retinal area are.

3. Explanation.—The explanation of the variation of visual acuity with illumination in terms of these two curves in Fig. 4 is then as follows. Beginning with the lowest illuminations, vision is a function of the rods. The number of rods which are active is very small; this amounts to having a resolving surface with the receiving elements

sparsely distributed. The retinal distance between two just discriminable contours must be large, and visual acuity is very low. As the

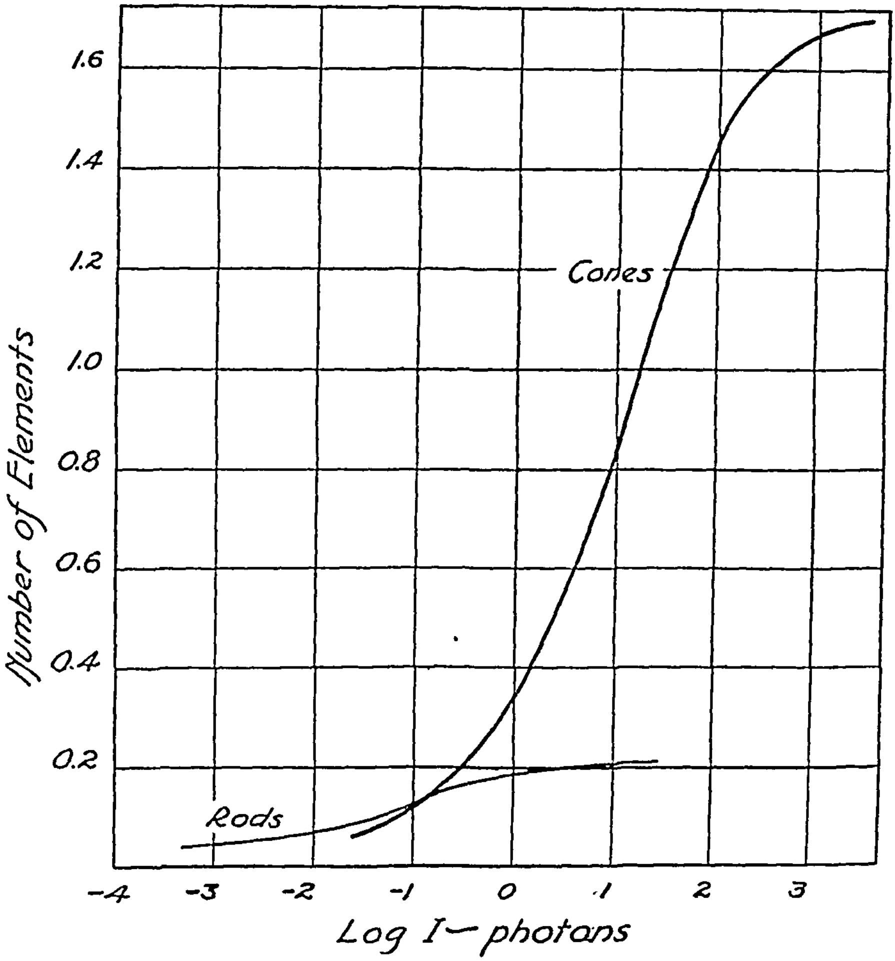

FIG. 4. Statistical distribution of sensibility of rods and cones. These curves are the integrals of those in Fig. 3, and give the relative number of elements per retinal area functional at any intensity. The curves may be described by the common Gram series of the statisticians. However, the equation here used is $KI = x^2/(a - x)$ where K and a have different values for the two curves as given in Tables I and II, and in Section IV. The ordinates read directly in units of visual acuity.

illumination increases, more and more rods reach their threshold and become functional. The average distance between the active elements becomes smaller, and visual acuity larger.

Presently an illumination is reached when the first cones begin to function. Visual acuity will still be mediated by the rods because there are already more functional rods present than cones. But as is apparent from Fig. 4, the rate at which the cones come into play with increased illumination is nearly ten times as great as the rate of the rods. Therefore at a certain point the number of cones functioning in the fovea will be equal to the number of rods in the periphery. As the intensity increases beyond this, the number of active foveal cones per unit area will be greater than the number of active peripheral rods in an equal area, and visual acuity will now be determined by the cones. This augmentation of the number of functional cones and the concomitant increase in visual acuity will continue until all the cones are active, and no further change in visual acuity is possible.

It is apparent that such a description of the relation between illumination and visual acuity is qualitatively acceptable. It remains to be shown that it is quantitatively adequate.

III.

Calculation of Normal and Color-Blind Visual Acuity.

1. Pupil Area.—The measurements of Koenig given in Fig. 1 were made with the natural pupil. Since the size of the pupil varies with the illumination, the sequence of intensities as given does not correctly describe the sequence of intensities falling on the retina. The outside illumination must be corrected by means of measurements relating brightness and pupil area. This correction will introduce a slight inaccuracy because it assumes that visual acuity is independent of pupil size. While this is not so (Cobb, 1914–15), it is better to neglect this slight source of error in comparison with the very large error which would result if there were no correction made for pupil area.

Fig. 5 gives the relation of pupil area to brightness calculated from the data of Reeves (1918, *b*). Reeves' illumination is given in millilamberts of brightness, whereas Koenig's data are in Hefner candles falling on white paper. Assuming a reflection coefficient of 0.8 (*cf.* Hecht, 1924–25),[2] one can convert Hefner candles into millilamberts by multiplying Koenig's values of I by 0.072. Koenig's data and

[2] Hecht (1924–25), p. 259.

Reeves' values for pupil diameter then become comparable and the illumination on the retina for any value of I in Koenig's data is secured by multiplying it by the corresponding pupil area at the brightness taken from Fig. 5. The pupil area is in sq. mm.; therefore the resulting retinal illumination is in photons (Troland, 1920).

In Fig. 1 there are 121 points. Their numerical values are given by Koenig in his original paper, and need not be reproduced in tabular

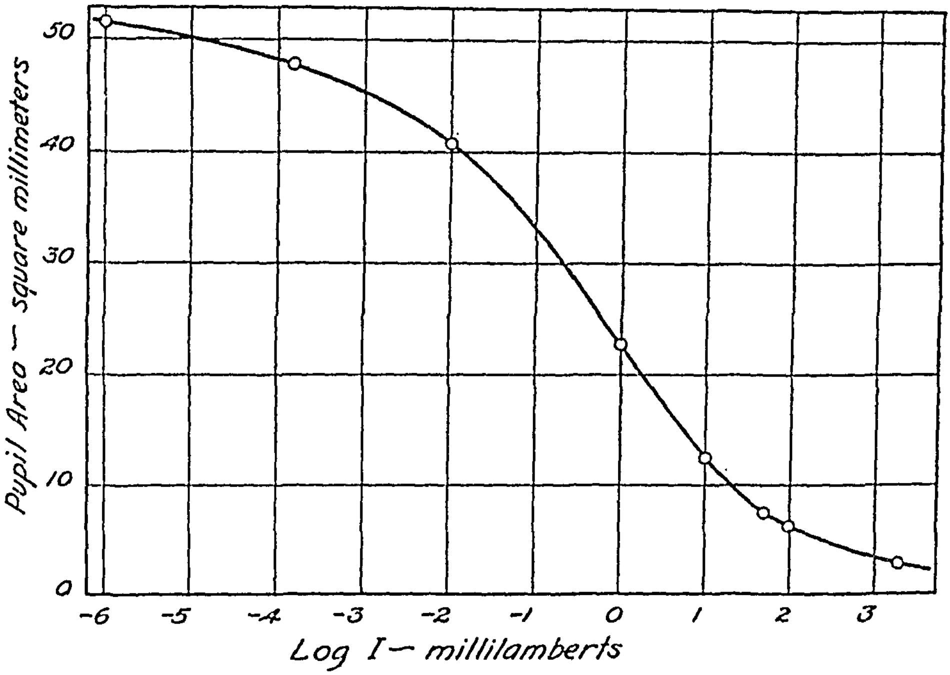

FIG. 5. Relation between pupil area and illumination calculated from the data of Reeves (1918, *b*).

form here. In working with them I have found it convenient to combine these data in consecutive groups of five readings, averaging their visual acuity and their log I values. Though not differing much from the average of the intensity values themselves, the average of the logarithms of the intensities is to be preferred because of the almost linear relation between log I and visual acuity. These averages are given in Columns 1 and 4 of Table I. In Column 2 are the pupil areas corresponding to the outside illuminations recorded in Column 1.

The retinal illumination is given in Column 3 as the products of Columns 1 and 2. Columns 3 and 4 then represent the relation between retinal illumination and visual acuity. It is these data that should be described by our assumption of the variability in threshold of the rods and cones.

TABLE I.

Relation between Illumination and Visual Acuity. Koenig's Data.

Outside illumination I	Pupil area	Retinal illumination I	Visual acuity, x: Observed average	Visual acuity, x: Calculated, rods $0.794\,I = \frac{(x-0.03)^2}{(0.21-x)}$	Visual acuity, x: Calculated, cones $0.0906\,I = \frac{x^2}{1.71-x}$
millilamberts	*sq. mm.*	*photons*			
0.0000297	49.6	0.00148	0.040	0.044	
0.000130	48.0	0.00625	0.068	0.058	
0.000390	46.6	0.0168	0.075	0.073	
0.000767	45.6	0.0350	0.093	0.087	
0.00269	43.5	0.117	0.13	0.12	0.13
0.00698	41.7	0.290	0.18		0.20
0.0112	40.3	0.451	0.22		0.24
0.0153	39.5	0.605	0.28		0.28
0.0214	38.5	0.826	0.31		0.32
0.0284	37.6	1.07	0.39		0.36
0.0397	36.5	1.45	0.45		0.41
0.0678	34.5	2.34	0.56		0.51
0.0826	33.8	2.79	0.61		0.54
0.137	31.9	4.36	0.72		0.65
0.215	29.8	6.68	0.75		0.76
0.585	25.4	14.9	1.00		0.99
0.980	22.7	22.2	1.00		1.10
1.47	20.7	30.4	1.12		1.19
3.57	16.5	59.0	1.30		1.38
8.93	12.9	115.0	1.44		1.49
26.6	9.3	248.0	1.61		1.62
65.9	7 1	468.0	1.69		1.65
155.0	5.4	838.0	1.68		1.66
1096.0	3.1	3400 0	1.71		1.70

2. *Normal Eye.*—Columns 5 and 6 give the values of visual acuity taken from the curves of Fig. 4 for the retinal illuminations given in Column 3. The correspondence between experiment and theory is seen to be well within the experimental error as judged by the scatter of the data in Fig. 1.

It should be pointed out that the curve for the rods in Fig. 4 begins not at zero, but at 0.030. If the curve were to begin at zero this would mean that the average distance between functional rods at the lowest perceptible illuminations is near infinity,—which is absurd anatomically. The value 0.030 for the lowest visual acuity is found by extrapolation from the data, and gives an adequate agreement with the other measurements. This correction of lowest visual acuity is not made for the cones, first because the cones do not function at their lowest values, and second because so small a correction as 0.03 may be neglected in the much larger values of cone visual acuity.

To show the relation between the computed values of Columns 6 and 7 of Table I and the original unaveraged and uncorrected data of Koenig, I have drawn the solid line in Fig. 1. This represents the computed visual acuities referred to the uncorrected intensisties of Column 1. The theory here proposed apparently gives an adequate description of these data. It is not without interest to see whether the recent data of Roelofs and Zeeman, given in Fig. 2, can be accounted for on the same basis. The solid line in Fig. 2 is drawn as in Fig. 1. It is clear therefore that the present theory forms an accurate basis for the explanation of the data of Roelofs and Zeeman as well.

3. Completely Color-Blind Eye.—In making the calculations I have assumed with Koenig that the lower limb of the data of Fig. 1 and of Fig. 2 represents rod vision, and the upper limb cone vision. Koenig recognized the implications which this involves, namely that in a completely color-blind individual the upper part of the curve should disappear and leave only the lower limb plus any extensions of it. He accordingly investigated the visual acuity of a completely color-blind person. The data he secured are given in the lower half of Fig. 6. They bear out his supposition that the lower limb is determined by the rods and the upper by the cones.

These data are similarly significant for us. In computing the lower part of the visual acuity data of Fig. 1, I have used only one-half of the rod population curve, on the supposition that at this point (*cf.* Hecht, 1924–25)[3] the cones overtake the rods in the number of elements which are functional. If this is correct, then the data for the visual acuity

[3] Hecht (1924–25), p. 252.

of the completely color-blind individual should extend to a distance beyond the inflection point of the normal eye, equal to that which has preceded it. Moreover the entire visual acuity data of the completely color-blind individual should be describable by the single rod distribution curve of Fig. 4.

Koenig's data are plotted in Fig. 6. With them are given the data previously obtained by Uhthoff (1886) for a similar case of color

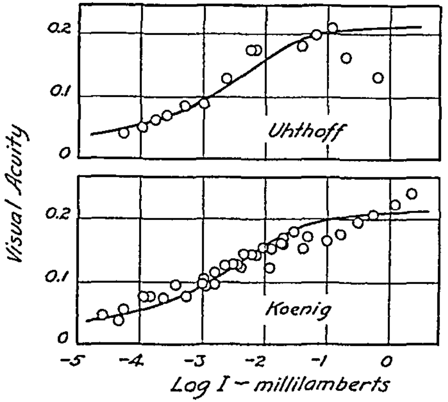

FIG. 6. Visual acuity of two completely color-blind individuals. Koenig's intensities have been multiplied by 0.072, and Uhthoff's by 0.000169 to convert them into millilamberts. In addition Uhthoff's units of visual acuity have been multiplied by 0.75 to render them comparable to Koenig's. Compare this figure with Fig. 1, but note that the ordinates here are twice as large as in Fig. 1.

blindness. To make Uhthoff's data comparable to Koenig's, the ordinates have been multiplied by 0.75. The smooth curve in both cases gives the values taken from the rod population curve of Fig. 4 but corrected for pupil area. Even though it is very likely that the behavior of the pupil is different for the color-blind eye, Reeves' values have been used again, because no other data are available. The details of the treatment are to be found in Table II, which gives, as

well, a comparison of the experimental data with those calculated in terms of the proposed explanation.[4]

IV.

Photochemical Basis of Sensibility Distribution.

1. Independence of the Statistical Analysis.—It is apparent from the previous section of this paper that the quantitative relation between visual acuity and the intensity of the illumination may be accurately described on the basis of a simple assumption, namely, that rods and

TABLE II.

Relation between Illumination and Visual Acuity of a Completely Color-Blind Person. Koenig's Data.

Outside illumination I	Pupil area	Retinal illumination I	Visual acuity, x	
			Observed average	Calculated $0.794\,I = \frac{(x-0.03)^3}{(0.21-x)}$
millilamberts	*sq. mm.*	*photons*		
0.0000546	49.0	0.00268	0.054	0.049
0.000361	46.7	0.0169	0.080	0.073
0.00132	44.8	0.0591	0.101	0.101
0.00359	43.0	0.154	0.131	0.130
0.00931	40.8	0.380	0.144	0.157
0.0254	37.9	0.963	0.166	0.181
0.0991	33.1	3.28	0.185	0.199
1.75	19.8	34.7	0.234	0.210

cones vary in their threshold sensibility according to the statistical manner of other populations. Because there are certain rather extended consequences to be derived from this idea, the method of its

[4] Since this paper was submitted for publication I have found in the literature another set of data describing the relation between visual acuity and illumination for a completely color-blind individual. The measurements are again by Uhthoff (Uhthoff, W., *Z. Psychol. u. Physiol. Sinnesorgan.*, 1899, xx, 326) with still another subject, but made after Koenig's work. These data of Uhthoff's furnish extraordinary corroboration of Koenig's values, since as Koenig showed (Koenig, A., *Z. Psychol. u. Physiol. Sinnesorgan.*, 1899, xx, 425) the two possess an almost point for point agreement.

presentation has been chosen so as to render the analytical treatment independent of any previous ideas concerning the nature of the retinal process.

This having been accomplished, it becomes desirable to show how this statistical distribution is derived from, and is, indeed, a necessary consequence of the photochemical system which I have previously used as a basis for the quantitative treatment of other properties of vision. In fact the present study of visual acuity is the effort at verification of deductions made from this photochemical system; and has acquired independence only secondarily.

2. Photochemical System.—It has been shown that the dark adaptation of the eye (Hecht, 1921–22), and its capacity for intensity discrimination (Hecht, 1924–25), are explainable on the assumption that in the rods and cones there is present a reversible photochemical system, whose general properties may be written in the form of the equation

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A \tag{1}$$

in which S is a sensitive substance, and P and A are materials which are decomposition products as well as precursors of S. Actually the reaction may be only pseudoreversible in that some other source of material or energy besides P and A are necessary for the regeneration of S. For the present purposes, however, equation (1) is adequate.

Let a be the total amount of S present in the system when there is no P and A; let light of intensity I shine on it for time t; and let x be the concentration of P and A formed and $(a - x)$ the concentration of S remaining. Then the velocity of the complete reaction will be

$$\frac{dx}{dt} = k_1 I (a - x) - k_2 x^2 \tag{2}$$

where k_1 and k_2 are velocity constants. At the stationary state of such a reaction $\frac{dx}{dt} = 0$ and

$$K I = \frac{x^2}{a - x} \tag{3}$$

where $\frac{k_1}{k_2} = K$.

Equation (3) describes the relationship between th[illegible] light to which the retina is exposed and the concen[illegible] ucts of photolysis of S present at the corre[illegible] of the photosensory system. The middl[illegible] relation between $\log I$ and x. The position [illegible] scissa axis depends on the value of the consta[illegible] curve, however, remains unchanged when K is [illegible] curve applies both to rods and cones, but the value [illegible] for the two (Hecht, 1924–25). This does not mean th[illegible] materials S, P and A are the same in the rods as in the cones, they may not be three different substances in the cones or in groups of cones. It does mean that the dynamics of the photosens[illegible] process, —its organization, —is the same in the two types of retinal cell.

The supposition is that P and A are "active" substances (chemically or electrically) and that the accumulation of a given concentration of them discharges the cell and sends an impulse down the nerve fiber. Very likely there is a secondary process intercalated between the accumulation of P and A and the discharge of the impulse (Hecht, 1925; Piéron, 1925), but that need not concern us at the moment. Suffice it that a given amount of P and A is required to cause an impulse to proceed along the attached nerve.

3. *Thresholds of Rods and Cones.*—Two assumptions may be made with regard to the amount of P and A necessary to discharge a cell. One is that the amount is the same for all the rods and for all the cones; the other, that the amount is different for the different rods and cones. In terms of the first, an increased concentration of these materials will then be indicated by an increase in frequency of discharge. This explanation serves as an adequate basis for intensity discrimination (Hecht, 1924–25) and agrees well with what Adrian and Zotterman (1926) have found for other sense cells. It is however inadequate as a basis for visual acuity because it supposes that all the rods and all the cones function at all intensities above the lowest thresholds. We know this cannot be true.

One must therefore adopt the other assumption,[5] namely that the

[5] There is, of course, the third possibility that the amount of P and A necessary for discharge is constant, but that the concentration of S varies widely in the

individual rods and cones differ in the concentration of P anc necessary to discharge them. Since there is no reason for prefen certain concentrations of P and A, it may be supposed that all concen tions of P and A have the same number of cells for which they spe cally represent the threshold of discharge. The rods and cones thus evenly distributed along the concentration axis (ordinates) of $x - \log I$ curve of Fig. 7; and the total number of elements which functional at a given value of I is directly proportional to the co sponding concentration x of P and A.

In relation to log I this linear dependence of the number of cells the concentration of P and A results in a distribution resembling integral curves of statisticians. In fact the curves of Fig. 4 are j this concentration-log I curve of Fig. 7. Moreover the skew freque curves of Fig. 3 are nothing more than the first differential $\frac{d:}{d \text{ lo}}$ of the photochemical curve of Fig. 7. Indeed the computations of theoretical values of visual acuity for Tables I and II, were made means of equation (2) relating concentration and I. The equat for the cones is

$$0.0906\, I = \frac{x^2}{1.71 - x}$$

and for the rods

$$0.794\, I = \frac{(x - 0.03)^2}{(0.21 - x)}$$

where x is visual acuity, 1.71 is the upper cone limit, 0.210 the up rod limit and 0.030 the lower rod limit. I is the intensity in photc

These upper and lower limits for rods and cones will vary for dif ent people, probably because the absolute number of retinal eleme may be different. But as is apparent from the comparison of the d

individual cells. My reason for neglecting this hypothesis at present is not t it may not be correct, but that preliminary calculations in terms of it have convinced me of its usefulness as a tool. One has to assume a rather awkward tribution of concentration among the cells in order to secure a distribution v regard to intensity which will satisfy the data of visual acuity. Neverthe this possibility may have to be explored some day.

of Koenig with that of Roelofs and Zeeman and with that of Uhthoff, the form of the equations remains adequate.

The agreement between experiment and calculation is thus much more significant than first appears. The frequency curves of statistics, both differential and integral, are largely empirical. They usually contain three or four parameters, and one can change them here and there in order to bring about an almost perfect fit. The curves in

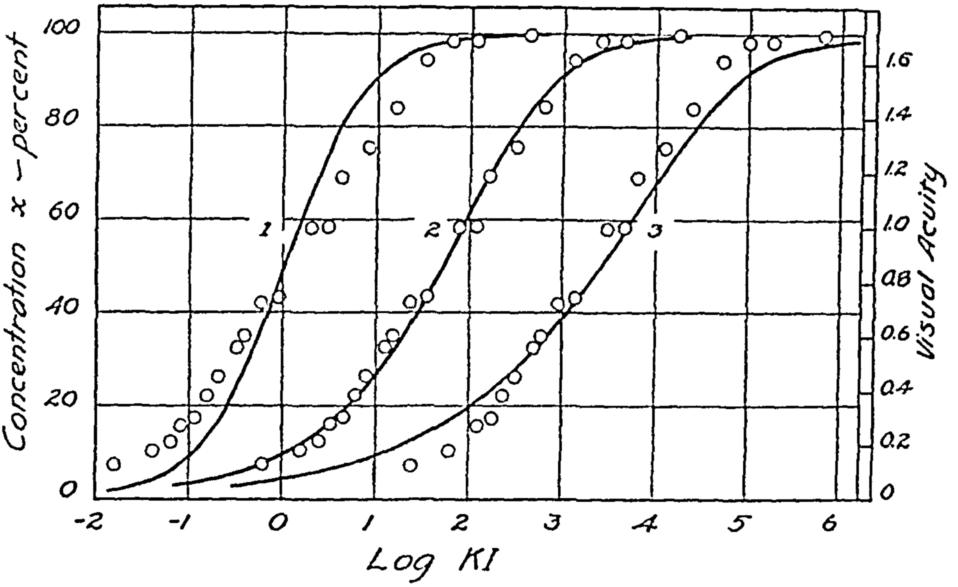

FIG. 7. This illustrates the relation of cone visual acuity to the stationary state curve $KI = \dfrac{x^n}{a - x}$ when n is 1, 2, and 3, and I is in photons. The visual acuity data are those of Koenig and are the averages used in Table I. It is apparent that the curve when $n = 2$ is the only one which describes the data adequately.

Figs. 3 and 4 are not derived in such a way. Their form depends on the properties of a photochemical mechanism, and is derived from previous knowledge of the behavior of the retina.

4. *Kinetics of the "Dark" Reaction.*—The equation of the stationary state (3), which is the basis for the curves describing visual acuity, is written on the assumption that the "dark" reaction which regenerates S is bimolecular. It has been contended (Lasareff, 1923, *b*) that this process need not be fixed as a second order reaction, but that

a first or third order reaction give results indistinguishable from that of a second order. This may be true for the kinetics of retinal dark adaptation, though there is evidence even here (Hecht, 1925), that such is not the case. Nevertheless, kinetic equations do not give an unequivocal statement of reaction order when actual concentrations are not concerned and arbitrary constants are freely available.

Fig. 7 shows three curves of the relation between x and $log\ I$ derived from

$$K\,I = \frac{x^n}{a - x} \tag{6}$$

where n is 1, 2 or 3, thus corresponding to the stationary state of a first, second and third order "dark" reaction respectively. The cone visual acuity data of Table I are plotted with each curve. It is apparent that a monomolecular "dark" reaction gives a curve which is too steep; and a trimolecular, one which is not steep enough to represent the data; whereas a bimolecular one corresponds accurately with the data. It may therefore be concluded that the recovery process in the retina is adequately described only by a bimolecular "dark" reaction.

V.

Related Theoretical Consequences.

1. Intensity Perception.—The assumption that the individual retinal elements have different "discharge" thresholds is sufficient as an explanation of the behavior of visual acuity with illumination, because it underlies the statistical relation between intensity and the number of functional elements per unit area. There are other properties of vision, however, which very likely take one other thing into account, namely the frequency of discharge of each cell. Consider, for example, the situation at an intensity when half the available cones are functional. The cones whose specific threshold is 50 per cent of P and A will discharge at a given minimal frequency. But the cones whose threshold is 25 per cent of P and A will discharge impulses along the nerve at twice this frequency; and so with the others. As the illumination intensity increases, two changes thus take place simultaneously in the retina. First, the number of elements functioning per unit

area increases; and second, the frequency of discharge of each of the already functional elements also increases. Visual acuity is attributable to the first of these changes. Other properties of vision such as intensity discrimination, intensity perception, and the recognition of flicker may depend on both of these changes.

In relation to the impulses travelling up the optic nerve, an increased intensity on a given retinal area will result in an increased frequency of nerve impulses, such as has been found by Adrian and Zotterman (1926) and Adrian and Eckhard (1927). This increase in frequency is due to an increase in the number of functional rods and cones and to an increase of discharge frequency of the already functional elements, because these two changes happen concomitantly.

It is well known that the same threshold effect may be produced by a small stimulus of high intensity or by a large stimulus of lower intensity (Reeves, 1918, *b*; Piéron, 1920). Intensity recognition would thus seem to depend on the total frequency of impulses in a group of closely associated fibers in the optic nerve, and may be produced either by many elements functioning in the larger area or by a few elements discharging at a higher rate in the smaller, more illuminated area.

The interchangeability of area and intensity cannot be exact. Since the spatial distribution of the thresholds of retinal elements within small areas is very likely fortuitous, the number of elements stimulated by a given intensity will vary directly with the size of the illuminated retinal area. On the other hand, the number of elements stimulated in a given area is an approximately logarithmic function of the intensity, as shown by Figs. 4 and 7. An accurately reciprocal relation between intensity and area can therefore exist only over a very limited range. This has been demonstrated by Reeves (1918, *b*) and more recently by Piéron (1920).

Such a description of intensity recognition is the logical extention of the all-or-none law to vision. This was first suggested perhaps by Brown (1913–14) and has had an interesting corroboration by Troland (1920). It is in harmony not only with what is known of the visual process but also of muscle contraction (Lucas, 1908–09), of nerve conduction (Adrian, 1913–14), of other sensory stimulation (Adrian, 1926), and of reflex contraction (Forbes and Gregg, 1915–16). It may be

deduced, as I have already pointed out (Hecht, 1924–25), eithe terms of a simple statistical distribution of sensibility, or as a co quence of a photochemical mechanism, and has no need for the ‹ fusing introduction of quantum theory speculation suggested Lasareff (1923, *a*).

2. *Unit Retinal Area.*—The term unit area has been used througl this paper, in a purely formal manner, as descriptive of the densit cone and rod population. Is there such a thing as a unit retinal a Is there a minimal retinal area which contains the equipment fo cording the various properties such as intensity perception, c vision, visual acuity, and the like, usually ascribed to the retina whole? One can, in terms of the ideas presented in this paper, m a calculation of the size of such a minimal retinal area.

As a direct result of his experiments with Brodhun on intensity crimination Koenig computed that it is possible to recognize 572 crete steps in brightness over the complete range of intensities ceptible to the eye. By comparing his computations with an anal of intensity discrimination (Hecht, 1924–25) it is apparent that first 30 of these steps are mediated by the rods; the remainder, 542, the cones. If the recognition of a minimal intensity difference re sents a change from n to $n + 1$ or to $n - 1$ in the number of co functional in a unit area, then the *minimal* retinal area in the fo must contain 542 cones. The actual unit area may be some multi of this; but the *minimal* area can contain no fewer than 542 co This number is thus derived directly from the experimental data intensity discrimination.

The minimal retinal area should also be able to mediate all vis acuities, from the highest to the lowest. Such an area will have its side the retinal distance between two just perceptible conto corresponding to the lowest visual acuity. The retinal distances all other values of the visual acuity will obviously fall within this ‹ tance. Moreover, this minimal retinal area for visual acuity is definition the same for rods and for cones, since the unit of vis acuity is taken to be the same for both. From Koenig's data in Fi 1 and 6, the lowest visual acuity is 0.03 units. Koenig's unit of vis acuity corresponds to a visual angle of 1′ 20″. Therefore a visual acu of 0.03 refers to an angular separation of slightly over 44′, and to a reti

distance of 0.2 mm. The resulting square area is 0.04 sq. mm. The fovea contains 13,500 cones per sq. mm. (Helmholtz, 1896[6]). The minimal area derived directly from visual acuity data therefore contains 540 cones, which is the same number as that derived from intensity discrimination.

From Figs. 4 and 6, it is apparent that in the normal eyes the cones take over the mediation of visual acuity from the rods when only half of the rods are functional. There must therefore be about 60 rods per unit area in the retinal periphery in order to account for intensity discrimination. This is a ratio of 1:9 of rods to cones. Note the same relationship in visual acuity. The maximum cone visual acuity is 1.71, the maximum rod visual acuity is 0.21. This is a ratio of 1:8+ of rods to cones.

This does not necessarily mean that the number of rods is actually only about 60 per unit area. Whereas the cones in the fovea are connected in a 1:1 ratio with optic nerve fibers, the rods in the periphery are connected in a much larger ratio to nerve fibers. Such a group of connected rods can have but a single local sign, and act as a unit in visual acuity. There are probably many more than 60 rods per unit area, the exact number depending on the ratio of rods to nerve fibers.

It should be pointed out that the ratio of rods to cones in a unit retinal area corresponds with the findings in the photochemical system suggested as a basis for the sensibility distribution of the retinal elements. For a minimal intensity discrimination it was found (Hecht, 1924–25) that in the cones this means an increase of 0.20 units of photochemical decomposition, whereas in the rods the increase is 1.6 units. Since there are 100 units in each photochemical system, there are possible 500 photochemical steps for the cones, and 60 for the rods; again a ratio of 1:8+ of rods to cones.

3. *Relation to Color Vision.*—It must be obvious that the assumption of statistical variations in the threshold sensibility of the rod and cone populations of the retina is capable of wide application in the physiology of vision. This relates particularly to color vision.

It has been frequently suggested (*e.g.* Troland, 1917) that the three "fibers" of Young's original color theory may be considered as three

[6] Helmholtz (1896), p. 260.

species of cones. Such a three cone hypothesis incorporates the tl fiber idea of Young and the three substance idea of Helmholtz. E type of cone contains its own photosensitive substance whose sorption spectrum can be derived from the *Grundempfindunger* Koenig and Dieterici (1892); and at the same time it is connected v an optic nerve fiber, stimulation of which produces in the brain the responding sensation.

This supposition of separate cones for the three different *Gi dempfindungen* is valuable because it enables one to extend to c vision the all-or-none law with all its realistic and clarifying coı quences. Formally, however, it is only slightly better than the oı nal fiber theory of Young, which also rests on Müller's doctrin specific energies. In order to convert the three cone idea into a us tool for the analysis of color vision there must be added to it the c cept which has helped us in our present study of visual acuity. must be assumed that the individuals of each of the three specie foveal cone are distributed with regard to their threshold sensibility a manner comparable to that which we have postulated for the co as a whole. In other words one must suppose that the cone curv Figs. 3 and 4 is a composite curve consisting of three identical cur each relating to one species of cone, and comprising about one-thir the cone population per unit area. The number of cones of a gi species which is functional per unit area depends on the relation gi by Fig. 4, where the intensity is determined by the amount of li absorbed by the corresponding sensitive substance at any wave-leng This depends on the absorption spectrum of the photosensitive sı stance in each cone species and may be derived from the three *Gr dempfindungen* curves of Koenig and Dieterici.

The implications of such an idea can be shown by a few exampl Consider for the moment only the matter of the number of con *Brightness depends on the total number of cones functioning in a u* area; and brightness discrimination corresponds to the addition or sı traction of one functional cone per unit area. Similarly color or h depends on the relative number of the three kinds of cones which functional in a unit area; and hue discrimination at constant brig *ness corresponds to the addition or subtraction of one functional cc of a specific type per unit area.*

If a given portion of the spectrum brings into play n_r, n_g and n_b number of cones of these types per minimal retinal area, then a portion of the spectrum which will cause a change from n to $n + 1$ or to $n - 1$ in one of these three types of cone will be discriminated by the eye as different in hue from the first portion of the spectrum. Moreover, since the three *Grundempfindungen* extend practically over the whole extent of the spectrum, the change from n to $n + 1$ in one cone species must mean an associated change in the other two species as well. And since the brightness of the monochromatic patches are usually held constant in such determinations, thus making the total number of functional cones constant, a minimal change in hue will *on the average* involve the simultaneous change of three cones, one of each species. We found that a minimal retinal area contains 540 cones. Granting three cone species of approximately equal numbers we get about 180 cones per species per unit area. If, now, a spectral hue discrimination involves *on the average* one of each species of cone, then the number of monochromatic patches obtainable with a spectrum is about 180. It is significant that Koenig (1894–95) obtained 160 such patches; Nutting (1920) between 130 and 180; Laurens and Hamilton (1923), 161 and 207 respectively.

Another example of the application of the statistical distribution of thresholds to color vision concerns the matter of complementary colors. Suppose that the sensation of white results in the brain when in a unit area there is functional a definite ratio (perhaps 1:1:1) of cones of the three types. A spectrum color cannot of course produce this ratio, but must involve a preponderance of that species of cone whose absorption maximum lies nearest this particular wave-length. Then the complementary of this spectrum color must be the one which will cause to function such a number of cones of the different types that the total number of functional cones of the three types will be in the ratio required to make white in the brain.

These bare essentials have been mentioned here, because they follow almost obviously from the original assumption applied to visual acuity, namely, that the thresholds of the cones and of the rods are distributed in relation to the intensity in a statistical manner similar to that of other populations. The quantitative details of these applications to color vision will be presented in future communications.

VI.

SUMMARY.

1. Visual acuity varies in a definite manner with the illumination. At low intensities visual acuity increases slowly in proportion to log I; at higher intensities it increases nearly ten times more rapidly in relation to log I; at the highest illuminations it remains constant regardless of the changes in log I.

2. These variations in visual acuity measure the variations in the resolving power of the retina. The retina is a surface composed of discrete rods and cones. Therefore its resolving power depends on the number of elements present in a unit area. The changes in visual acuity then presuppose that the number of elements in the retina is variable. This cannot be true anatomically; therefore it must be assumed functionally.

3. To explain on such a basis the variations of visual acuity, it is postulated that the thresholds of the cones and of the rods are distributed in relation to the illumination in a statistical manner similar to that of other populations. In addition the rods as a whole have thresholds lower than the cones. Then at low intensities the increase in visual acuity depends on the augmentation of the functional rod population which accompanies intensity increase; and at higher intensities the increase in visual acuity depends on the augmentation of the functional cone population. The number of cones per unit foveal area is much greater than the number of rods per unit peripheral area, which accounts for the relative rates of increase of rod and cone visual acuity with intensity. At the highest illuminations all the cones are functional and no increase in visual acuity is possible.

4. If this division into rod visual acuity and cone visual acuity is correct, a completely color-blind person should have only rod visual acuity. It is shown by a study of the data of two such individuals that this is true.

5. The rod and cone threshold distribution has been presented as a purely statistical assumption. It can be shown, however, that it is really a necessary consequence of a photochemical system which has already been used to describe other properties of vision. This system consists of a photosensitive material in reversible relation with its precursors which are its products of decomposition as well.

6. On the basis of these and other data it is shown that a minimal retinal area in the fovea, which can mediate all the steps in such functions as visual acuity, intensity discrimination, and color vision, contains about 540 cones. Certain suggestions with regard to a quantitative mechanism for color vision are then correlated with these findings, and are shown to be in harmony with accurately known phenomena in related fields of physiology.

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THE MEASUREMENT OF GALVANOTROPIC EXCITATION.

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I.

The execution of galvanotropic orientation is in many cases precise, under proper conditions, and patently escapes the possibility of adaptive complications. It is therefore a desirable tool for use in attempts toward analysing behavior through the synthesis of situations in which conduct is determined by several types of concurrent excitation (*cf.* Crozier, 1925–27, 1926–27; Crozier and Pincus, 1926–27; Crozier and Stier, 1927–28; Wolf and Crozier, 1927–28). From a slightly different approach, the measurement of galvanotropic excitation presents difficulties. It is not easy to see a way in which the relationship between tonic excitation by constant current and the energy of the current can be directly expressed. The relation of tissues and cytoplasmic surfaces to constant current are not yet sufficiently clear (*cf.* Osterhout, 1927). Tests preliminary to an intended attempt of this sort, employing the slow curvature of roots, have shown the common conception of this particular orientation to be based upon an imperfect understanding of the experimental conditions (Navez, 1926–27). Indirectly, however, it may be possible to obtain the expression of true galvanotropic excitation in energy terms, by means of a procedure which also illustrates the resolution of conflicts in orientation. The procedure is similar to that used in experiments involving the compounding of phototropic and geotropic orientations (Crozier and Pincus, 1926–27; Wolf and Crozier, 1927–28). The results here given are preliminary, in the sense that they are an illustration of the outcome of the method rather than a development of its possibilities.

II.

The triclad *Leptoplana variabilis* is cathodally galvanotropic. It is also photokinetically reactive, and negatively phototropic. The negative phototropism makes it possible to arrange a field of excitation in which the phototropic vector component is at right angles to

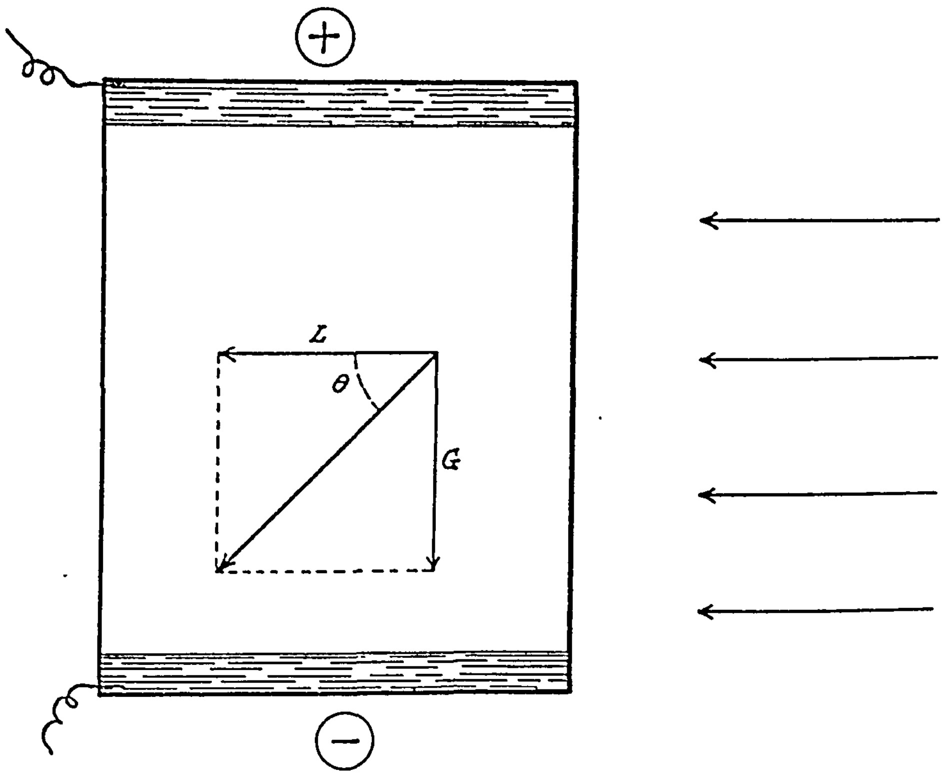

FIG. 1. Diagram of experimental trough with cotton lead-in pads (lined), for establishment of constant current giving rise to the galvanotropic vector, *G*. Light from the right side (arrows) is responsible for the phototropic vector, *L*. The galvanotropic excitation is equivalent to the phototropic when the resultant path makes an angle $\theta = 45°$.

a simultaneously imposed galvanotropic vector (Fig. 1). By varying the intensity of the acting light, it should be possible to find, at each of several intensities, a density of current just equivalent in orienting efficiency. This should result in creeping along a path at an angle of 45° to that pursued when the light alone is acting, or, on the other axis, to that enforced by the current in darkness or under non-directive

illumination. Qualitative experiments showed that this type of effect is certainly found. Other arrangements are also possible; thus the light might be allowed to act in a direction parallel to the lines of current flow, and another balance-path chosen as reference line or end-point. But in such tests as we have thus far made the procedure first outlined has given the most continuous creeping. To avoid the disturbing stimulation induced by sudden changes of light intensity, and also the trouble of maintaining reasonable constancy in the galvanic current, it is of course perferable to make the light intensity the independent variable, and to match its influence on orientation by controlling the current density.

The measurements involve difficulties. The triclads should be freshly collected, and (at this locality) should not have been exposed for any length of time to temperatures above 20°C., which is also an upper critical temperature for normal behavior and activity in a number of other animals from the same general habitat (*cf.* Crozier and Stier, 1927–28). Prolonged light adaptation must be guarded against, and excessive galvanic stimulation must be avoided, else injury or at least temporary failure to continue creeping certainly results. The maintenance of phototropic and galvanotropic excitability, which may appear to be depressed for brief periods, must be separately tested at intervals during the measurement, and orientation checked by reversing the current; possible guidance by preceding slime trails on the glass must be obviated by frequently cleaning it.

The experiments were made in a dark room, the observation stage being a large, black rubber developing tray with thick cotton wads across each end, through which the current was led from a bank of dry cells. With sensitive milliameters and voltmeter the current density and voltage were controlled by a sliding rheostat and suitable switches. A glass plate fitted the bottom of the tray and was marked on its under surface with 45° lines in red. Planarians tested for galvanotropic and phototropic orientation were allowed to orient directly away from the light, and then by gradually increasing the current density a point was found at which a path was persisted in parallel to or directly above one of the red lines, for about 6 cm. Slightly increasing the current density above this value led to creeping more directly toward the cathode. Owing to slow polarization at the electrodes, continuous adjustment of

the rheostat was required, using the planarian as a galvanometer. The reading of the instruments by the other observer gave a good check on the independence of the measurements. Care was taken to avoid reflection of light from the person or clothing of the observers and from the surrounding apparatus. Reflection and refraction due to air bubbles beneath the glass plate must be avoided by excluding such bubbles. Slight variation in the lighting current could not be controlled, but the data do not pretend to a precision such that this is of any significance. Light was obtained from a group of three 50 watt internally frosted bulbs arranged in a line and mounted on a stand in such a way that the whole surface of the planarian was illuminated at a slight angle. By intermittent immersion of flasks containing ice

TABLE I.

Relative Intensities of Illumination, Given in Arbitrary Units ($10^6 \times$ Inverse Square of Distance of Source in Cm.), and the Corresponding Mean Current Densities (Milliamperes per Sq. Cm.) Required to Balance the Phototropic Orientation by Galvanotropic, with the Probable Errors of the Average Readings (Which Are 2.9 to 3.5 Per Cent of the Means).

Light intensity	Current density	Light intensity	Current density
323.2	1.57±0.047	76.66	0.954±0.027
137.8	1.23±0.039	22.74	0.495±0.018

the temperature was kept between 16° and 18°C. At 20° or above, creeping and orientation become erratic.

III.

Data from a series of repeated tests with four individuals, during 1 day, are collected in Table I. The light intensities (in different experiments, from 20 to 400 meter candles approximately) are relative, being given as the inverse squares of the distances from the axis of the lamps to the center of the creeping plate. The current density, with P.D. = 0.2 to 0.4 volt, had to be varied between 0.44 and 2.67 ma./sq.cm. The tests were rotated haphazardly from one intensity to another. The separate readings showed good qualitative agreement, as indicated by the probable errors of the means, and it was clear

that the current density required to balance the orienting effect of the light increased in a regular way with the intensity. When plotted (Fig. 2), the relationship appears to be such that the current density is directly proportional to the logarithm of the light intensity.

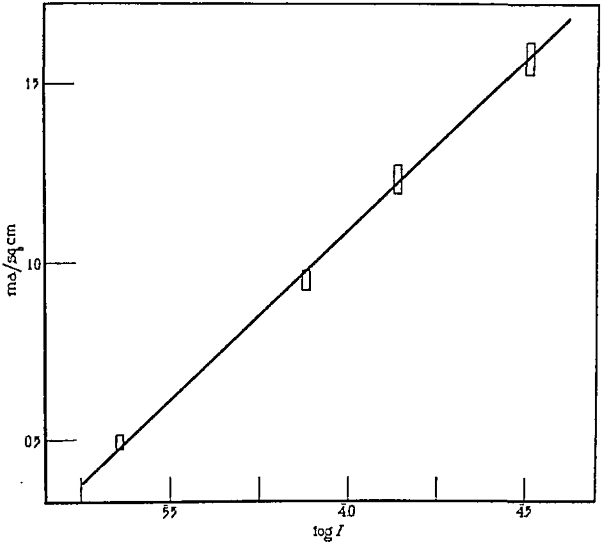

FIG. 2. The current density in milliamperes per sq. cm. is directly proportional to logarithm of the relative intensity of the light, for orientation at 45° to the path under either mode of stimulation alone. The observations are plotted as bars centered on the mean current densities and having a vertical height equal to twice the probable error. Data in Table I. (Experiments with other individuals showed that this graph may hold up to intensities 100 times as great as the highest employed in the present experiments, but it is difficult to secure continuous series of readings.)

If the photic excitation be taken as proportional, over the range employed, to *log I* (*cf.* Hecht 1919–20; Crozier, 1925–27; Crozier and

Pincus, 1926–27; Wolf and Crozier, 1927–28), then the galvanotropic excitation must be held directly proportional to the current density. This conclusion obviously rests upon the validity of the assumption that the two modes of excitation operate by producing differential tonic contractions of the orienting musculature on the two sides of the body. When the photically induced tonus on one side is equalled by the cathodally induced tonus on the opposite side, orientation ceases. This is tested in two ways. If, at any moment during the resultant creeping, one or the other excitation ceases, full normal orientation as governed by the remaining force is at once in evidence. Also, if the light intensity be made quite high, and the current density correspondingly increased, the planarian shortens and may cease to creep, although change of current density continues to evoke corresponding turnings of the anterior end; the total effect on tonus is therefore additive. There is consequently no ground upon which to avoid the conclusion that each kind of excitation is continuous when creeping is oriented in the compound field.

It may be noted that the "law of the resultant" takes for this case a form slightly different from those usually encountered. Its complete expression must await experiments in which the angle of orientation is measured when both current density and light intensity are varied.

SUMMARY.

The expression of galvanotropic excitation in energy units is obtained by the measurement of the current densities required to balance phototropic excitation (or reciprocally). With the triclad *Leptoplana* preliminary measurements show that the current is proportional to the logarithm of the light intensity.

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ON THE PLACE OF PHOTIC ADAPTATION.

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(Accepted for publication, October 1, 1927.)

I.

As a method for the investigation of photic adaptation in a phototropic organism we have employed the measurement of phototropic divergence from the vertical path of geotropic creeping of the slugs *Agriolimax campestris* upon a perpendicular plate, the source of light being at one side (Wolf and Crozier, 1927-28). This divergence decreases with time, and in a manner easily accounted for by the simplest assumptions. The point we now discuss concerns the assumption that the decreasing effectiveness of light in driving the animal away from its course in darkness is due to photic adaptation of the eye. Clearly, there are at least two general possibilities in such a case. Either the continuous interplay of the inner (central) mechanisms respectively concerned with the geotropic and with the phototropic orientations results in the gradual establishment of a condition which internally "blocks" impulses arising in the eye; or else the photic irritability of the eye declines with continued exposure to light. The manner in which the behavior simulates "learning" is worthy of note.

It is important to obtain some means of deciding between the two alternatives, on the one hand of a (presumptively central nervous) adjustment resulting from competitive excitations, on the other a purely peripheral sensory adaptation.

The test relied upon to further a decision is of a very elementary kind. It consists in comparing the forced angular divergences from vertical creeping in the case of (1) slugs exposed to light while on a vertical surface, as in the experiments previously discussed, and (2) exposed to light of the same intensity while on a horizontal surface,

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until the moment of testing. While it might be thought necessary to study also the rôle of adaptation or exhaustion with respect to continuing geotropic excitation, we find it not imperative to do so,—because continued successive tests of geotropic orientation yield, within the limits required, no evidence of geotropic fatigue; and especially because the waning influence of light, with continuous exposure, leads to greater and greater amplitude of the geotropic response. The required evidence, therefore, must show whether the extent or the apparent rate of photic adaptation is due in any way to the concurrent geotropic excitation. When under the conditions of these tests the slugs creep vertically, with no detectable influence of the lateral light, they still (for a time) orient when illuminated upon a horizontal plane (as when the creeping plate is lowered). This signifies, as we have already intimated (Wolf and Crozier, 1927-28), that a sort of threshold must be recognized for the balancing of the phototropic effect by a geotropic one; its nature can be investigated by means of observations at different inclinations of the plane of creeping, but it in no way interferes with the proposed test.

The experiments were made by placing slugs, dark-adapted over night or longer, upon a moistened plate of ground glass. The plate was horizontal, light falling upon one side of the animal. By gentle rotation of the plate, keeping pace with the negatively phototropic orientation, light was maintained upon that side for a known time (estimated by a stop-watch). The plate was then tilted so as to be vertical, and the angle of orientation during creeping is charted exactly during the next 0.5 minute. As in previous experiments (Wolf and Crozier, 1927–28), the angle of deviation from vertical, measured to 0.5°, is then considered to be that appropriate to the mid-time of execution of the trail. By suitable spacing of the intervals of exposure, data are obtained in successive trials with each of a number of individuals. Occasionally it is necessary to start the animal creeping by stroking it with a moist camel's hair brush. Such manipulation does not affect the amount of orientation. The temperature of the dark room was 20–21°.

II.

After a variety of preliminary trials, which gave results in no way inconsistent with those here recorded in detail, a final experiment was

made with 81 individuals. The intensity of the testing light was constantly 29.4 f.c. Table I contains the mean angles of orientation (β) away from the vertical, with their probable errors, after different intervals of exposure to light upon a horizontal surface. During the time elapsing up to the moment of testing, therefore, there can be no question of adjustment as between geotropic and phototropic impulses. It was previously shown that for photic orientation during light adaptation, in which the phototropic vector decreases in magnitude, the angle β should be expected to vary in such a way that the logarithm of its tangent decreases linearly with time (Wolf and Crozier, 1927–28).

TABLE I.

The angle of departure of *Agriolimax* from straight upward creeping upon a vertical plate illuminated from one side decreases with exposure time. Data from experiments with 81 individuals, the creeping plane being horizontal except during execution of the trails from which β is measured. Orienting light = 29.4 f.c. The calculated values are obtained from the curve fitting earlier data secured under continuous geotropic excitation.

Exposure time	β found	β calculated
min.		
0.5	63.0 ± 0.911°	65.2°
1.0	58.3 ± 1.19°	57.6°
2.0	42.6 ± 1.85°	41.0°
3 0	22.8 ± 1.06°	24.2°
4.0	14.1 ± 1.35°	13.7°

It is quite apparent that this relationship is accurately obeyed in the present case also, as shown by Fig. 1. The formula expressed by the graph in Fig. 1 results from the assumptions (1) that the geotropic effect is constant and (2) that the phototropic effect decreases exponentially with time. The nature of the present result indicates that the general character of the photic adaptation is independent of concurrent geotropic excitation.

The analysis can be carried further. It was shown that the *rate* of photic adaptation in these slugs is independent of season, and that data secured from separate lots of individuals under comparable conditions agree quantitatively (Wolf and Crozier, 1927–28). Such comparisons are facilitated in a practical way by the fact that the rate

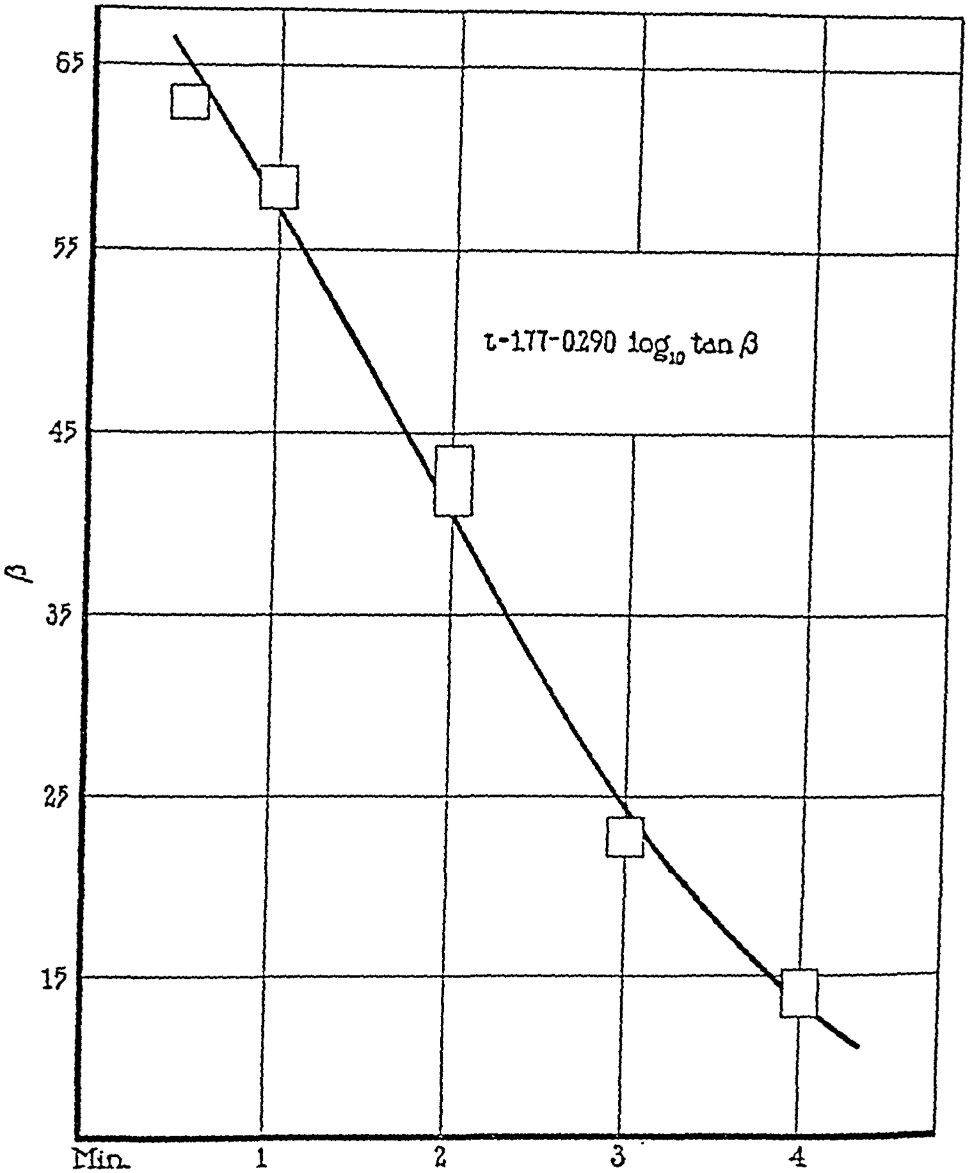

FIG. 1. The angle of orientation of *Agriolimax*, the departure (β) from a vertical path upon a vertical plane as forced by the phototropic effect of light from one side, after increasing periods of exposure to illumination of 29.4 f.c. The slugs were creeping upon a *horizontal* surface except during the half minute required to obtain the orientation trail. The observations are plotted as bars centered on the means, with height = 2 P.E. The curve is that of the equation

$$\text{time} = 1.77 - 0.290 \log_{10} \tan \beta,$$

and the agreement with the observations shows that, as found previously (Wolf and Crozier, 1927–28), log tan β decreases linearly with time.

of light adaptation is practically independent of temperature (within the range 15° to 22°). Legitimate comparison may therefore be made between the measurements of the present series and those already

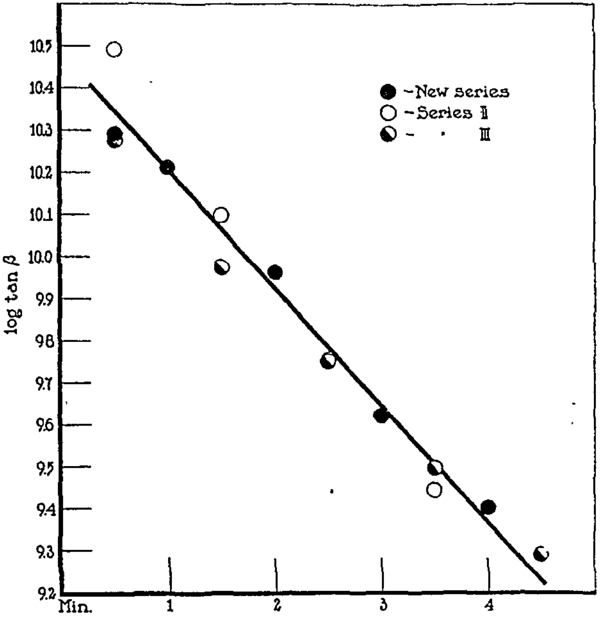

FIG. 2. Data from two earlier series (II, III) of experiments are plotted together with those of the present paper, to demonstrate the agreement in the extent and in the rate of photic adaptation, and thus their independence of continuous concurrent geotropic excitation, as practiced in Series II and III.

given in the previous paper for adaptation to light of the same intensity. It is evident from Fig. 2 that the two series are in excellent agreement, not only in their general course but also in every quantita-

tive detail, including the important matter of the *rate* at which adaptation takes place. In fact, the curve in Fig. 1 is that obtained from the earlier observations.

III.

That adaptation to repeated or continued excitation is a matter of the sensory receptors concerned may seem to be so obvious as not to require proof. Yet a moment's consideration will show that in phenomena of phototropic excitation such as that we have considered, the conclusion is by no means self-evident. The manner in which, particularly in the presence of other modes of excitation, one form of response appears to gain ascendency over another clearly simulates the type of adjustment commonly evident in "learning." If it can be shown, as in the present instance, that the "adjustment" occurs in the absence of the competing stimulation, there is no recourse but to place the site of adjustment, not in the central nervous organ, but in the receptors. The case is thus somewhat different from that treated by Hecht (1918, 1918–19, 1922–23), in which it was shown that adapting changes in the responses of *Ciona* and *Mya* to repeated excitation by light are satisfactorily and quantitatively accounted for by the properties of a simple receptor mechanism, without appeal to "higher behavior." The difference lies in the fact that with *Agriolimax* there is involved the added complexity of another form of excitation, and the possibility of central nervous competition therefore given; from the nature of the phenomenon, moreover, it is possible to deal with the time factor in such conceivable competitions, which in certain earlier experiments (Crozier and Pincus, 1926–27) could not be managed.

SUMMARY.

The progress of photic adaptation of *Agriolimax*, when studied by the method of compounding phototropic and geotropic vectors, is shown to be uninfluenced by the concurrent gravitational excitation. Direct proof is thus obtained that the adaptation to light, manifest in its steadily decreasing effectiveness as a stimulus during the course of exposure, is not due to any central nervous adjustment simulating "learning," but is due to photochemical changes in the receptors.

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PHOTOTROPISM OF DIXIPPUS MOROSUS.

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Axenfeld (1899) found that there is a local difference between the upper and under part of an eye in the effect of stimulation by light. Since his experiment Loeb (1918), Garrey (1917), Mast (1911) and other authors have mentioned the same phenomena regarding different orders of insects. So far as I have found, the local difference in the effect of light is considered to be a matter of upper and lower regions, or of anterior and posterior of the eye. The writer used a walking-stick (*Dixippus morosus*) to study these local differences in a compound eye, and for measurements of phototropism.

The compound eyes are parallel, side by side of the genæ, making no perceptible angle between the planes of the eyes. So we can infer that an angle of orientation made by the body corresponds to the angle at the base of the eye. When the right eye is illuminated after covering the lower half of the left eye, the insect orients toward the *right*, making a circuitous movement. If the light comes from above, the insect orients toward the *left* with a circuitous movement. The latter orientation is also shown when the illumination is from underneath the body, either from the front or the back.

I have found that if the peripheral part of the right eye is covered with lamp black, and the insect is placed in a room which is lighted with either the usual daylight or diffuse artificial light, that it will orient toward the *right* side as it does under the illumination at a right angle from the side. But the insect orients toward the *left* side when the central part of the eye remains uncovered.

From these investigations we conclude that the local differentiation for reception of light is apparent when the eye is uniformly acted upon by light evenly distributed; the facets at the peripheral part of the

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eye of *Dixippus* appear to be of equal significance for reaction to light. Such local difference may be related to the life of this insect, which rests upon the stem of the plant being lighted from above and beneath, or from front and behind the body.

Experiments with Dixippus Placed upon a Vertical Surface.

Dixippus walks directly up a vertical surface or rests with the body axis in a vertical line unless stimulated by light from one side. When the insect is illuminated from one side of the body it no longer walks vertically but veers off at an angle toward the source of the light. The lower limit of non-effective intensity of the light is about 0.7 foot

TABLE I.

Mean Angle of Orientation (θ) at Different Intensities of Light (Foot Candles); the Probable Error of Each Angle (P.E.); and the Coefficient of Variation of θ (C.V.).

Intensity of light	log I	θ	P.E.	C.V.
foot candles				*per cent*
1.2	0.0792	88.1°	±0.2209°	1.95
1.7	0.2304	73.16°	±0.2379°	2.95
2.9	0.4624	63.84°	±0.2304°	3.60
4.7	0.6721	53.64°	±0.2804°	5.15
8.6	0.9345	43.84°	±0.2456°	6.41
17.0	1.2304	35.2°	±0.2428°	6.60

candle. The angles of inclination of the body, at rest, toward six different intensities were observed in the dark room on five individuals. Five measurements of an angle of orientation toward each intensity of light were made on each individual.

The results summarized in Table I are averages of twenty-five tests at each intensity of light upon the vertical plane. The experiments tell us that the angle of orientation toward the light varies inversely with the intensity of the light when the insect is illuminated from one side upon a vertical plane.

It can be shown that the cotangent of the angle θ (Fig. 1) is proportional to the logarithm of the intensity of the light. In terms of the theory of orientation under such conditions (Wolf and Crozier, 1927–28) the position of orientation should be predicted by the force diagram

in Fig. 1. Since adaptation of *Dixippus* to light is slow, the angle θ may be studied as a function of the intensity of the acting light, I. The linear form of the body of the insect makes it particularly suitable for such measurements, which are further facilitated by the maintenance of a position of orientation without creeping. If, as is commonly

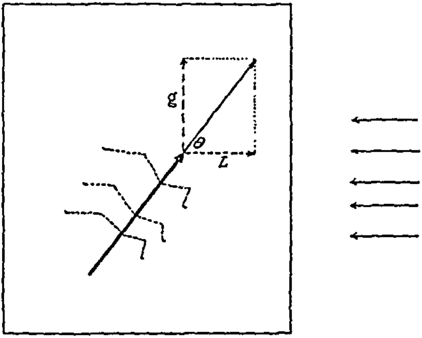

FIG. 1. Position of orientation assumed by *Dixippus* on a vertical surface (covered with black cloth, giving a firm hold for creeping), when illuminated from the right side. The posture of the legs is determined by differences in extensor tonus comparable to those seen in *Ranatra* (Crozier and Federighi, 1924–25). The angle θ is determined by the relation

$$\cot \theta = \frac{L}{g},$$

where L = the phototropic vector, g the geotropic. Since g is assumed constant (the plane being vertical), and L is taken as proportional to $\log I$, it should follow that $\cot \theta$ is a linear function of $\log I$ (see Fig. 2).

the case, photic excitation should be proportional to the logarithm of the intensity (over an intermediate range), then from Fig. 1

$$\Delta \text{ cotan } \theta / \Delta \log I = \textit{constant},$$

since the geotropic stimulation is constant. The graph in Fig. 2 gives a picture of this relationship, which is satisfactorily obeyed. The variability of θ as measured increases in the same way as θ decreases.

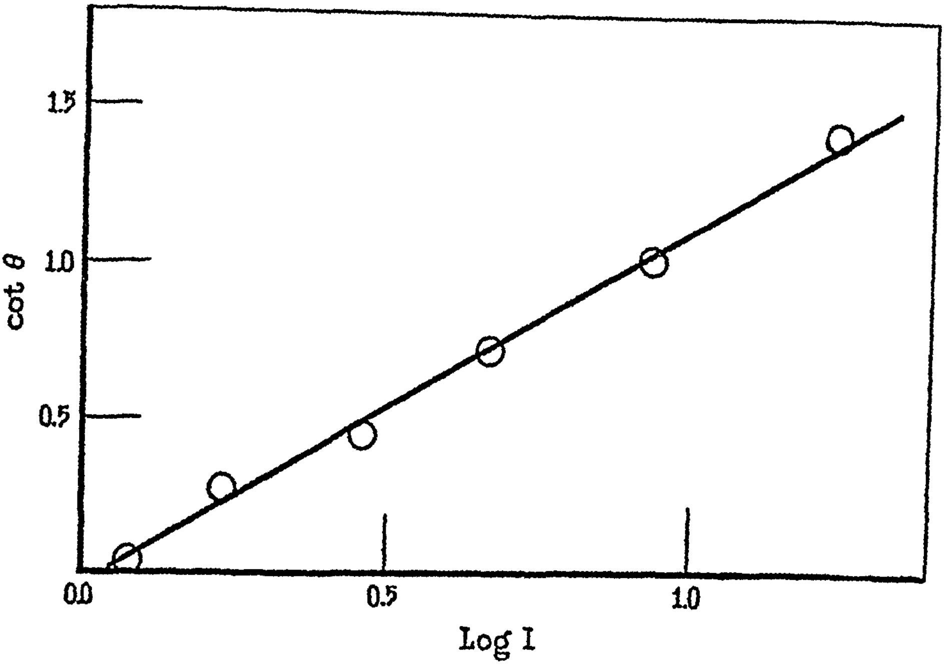

FIG. 2. The cotangent of the angle of inclination of the body of *Dixippus*, on a vertical surface with illumination from one side, is a linear function of the logarithm of the light intensity.

SUMMARY.

1. Local differences in the effects of stimulation of parts of the eye by light are expressed in *Dixippus morosus* by differential circus movements.

2. The angle of inclination of the body axis toward one source of light when the animal is on a vertical plane with light from one side is inversely proportional to the logarithm of the intensity of the light.

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THE SURFACE TENSION OF PHYSIOLOGICAL SOLUTIONS.

Difficulties of Measurement and Interpretation.

By J. M. JOHLIN.

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(Accepted for publication, October 24, 1927.)

A considerable number of publications in recent years have had the object of indicating the nature of surface tension phenomena in the case of physiological solutions, and many attempts have been made to find a clinical application for the surface tension measurements of such solutions. The peculiar difficulties of measuring the surface tension of semicolloids and of interpreting the data obtained have however caused the introduction of many errors.

It is well known that when, in the case of true solutions, a new interface is formed, a measurable period of time is required to bring about equilibrium following a change in the surface concentration of the solute, and that, in the case of semicolloids, hours or days may elapse before a condition of surface equilibrium is reached. In measuring the surface tension of semicolloids, it is this state of affairs which limits the choice of surface tension methods and which makes difficult the proper interpretation of the data which are obtained. Data obtained as apparent equilibrium values, even in the case of pure substances of a semicolloidal nature, are furthermore difficult to interpret because they usually are not a simple function of the concentration of the solution.

Because of the length of time required for surface equilibrium to be reached when the surface tension changes with time, only so called static methods can be employed for measuring the surface tension of semicolloids.* Of these methods, that of the sessile drop is the only

* This does not include cases when it would be desirable to measure the surface tension during that initial period in which the amount of surface concentration is not sufficient to bring about a change. Lord Rayleigh first showed that a measur-

one with which the angle of contact can be neglected. Unfortunately the progressive changes that it is frequently desirable to measure are with this method so small that they are within the limits of error of the method. The capillary rise method is, therefore, the only one that makes it possible to obtain successive readings with the same interface and to note how the surface tension of a particular interface is changing with time. This does not imply that it is possible by this method to obtain absolute values in all cases, and various criticisms of this method readily suggest themselves.

The ring method has been widely used and has been considered by some to be "the only device by which the surface tension of colloidal liquids can be accurately determined" (1). Lenard has been quoted (2) to help strengthen this point of view. Lenard (3), however, states that the straight wire method, "as opposed to the ring method," can be considered reliable. Even in the case of the straight wire this claim to accuracy should not be applied where thick surface films are formed and the surface tension keeps on changing for a considerable period of time. When a ring or a straight wire is lifted from the surface of a solution so as to determine the force that is required to rupture the film, the surface is considerably enlarged by the elongated film that is formed in the process of lifting, hence the surface concentration and consequently the surface tension cannot be the same as it was at the flat surface.

Another doubtful point is that of considering the surface effect of thick surface films, of a semisolid nature and visible to the naked eye, as surface tension phenomena in the same way that one considers the excess surface concentration in the case of true solutions where the material concentrated at the interface is in more perfect equilibrium with the bulk of the solution. These thick films are suggestive rather of a new phase than of a surface excess affecting the surface tension of an interface.

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able period of time must elapse before a quantity of the solute is adsorbed at a freshly formed interface in sufficient amount to appreciably alter the surface tension. The method which he used for determining this fact would have to be applied for surface tension measurements in such cases (Rayleigh, J. W. S., *Proc. Soc. London*, 1889–90, xlvii, 281).

In the present investigation it was shown that even in the case of pure liquids where surface equilibrium is almost instantaneous and consequently not appreciably disturbed by changing the form of the surface, *the ring method is not to be relied upon. The surface tension* of water was measured by the ring method at 0° and at 25°C., and that

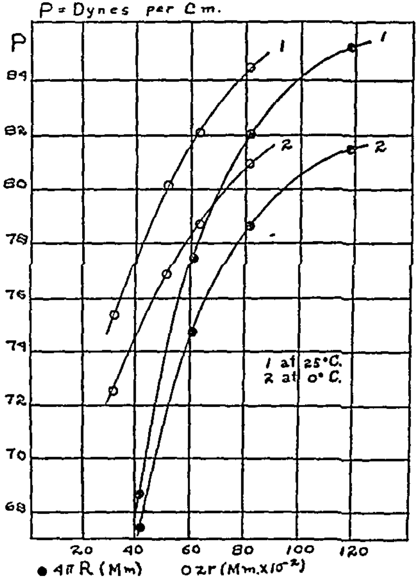

FIG. 1. Shows how the pull (P) required to detach the ring varies with the length of the surface ($2 \times 2\pi R$) on which it acts, *i.e.*, twice the circumference ($2 \times 2\pi R$). Determination with water at 0°C. and at 25°C.

of pure benzene at 25°C. Two sets of platinum rings were used with the du Noüy type of tensiometer. In one set of measurements the diameter of the ring was varied and in the other the size of the wire was varied while the rings were of the same diameter. The rings were

measured with a micrometer caliper after they had been snugly mounted on a tapering piece of machined steel. It is to be noted in Fig. 1 that as the length of the surface, $2 \times 2\pi R$, on which the pull is exerted is increased from 41 to 118 mm. (*i.e.*, as the diameter of the ring is increased from 6.5 to 18.8 mm.), the value obtained for the surface tension of water increases almost 25 per cent, and that the variations obtained as the size of the wire varies, though the diameter of the rings is kept nearly constant, are not quite as great. In Fig. 2, it is to be noted that in measuring the surface tension of pure benzene the variations were of the same nature though not as

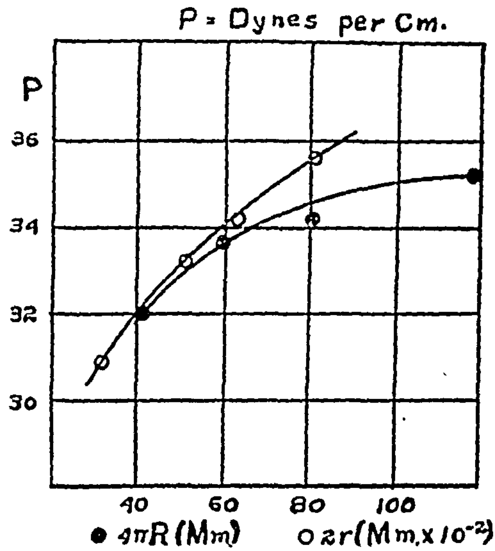

FIG. 2. Units same as those of Fig. 1. Benzene at 25°C.

great as in the case of water. All of these results are reproducible within a few hundreds of a dyne. These data confirm the results of Sondhaus (4) who first introduced the ring method 50 years ago, and who showed that there are large variations in the results obtained when the size of the ring is varied. Edser (5) has also reported similar results which showed that values obtained by this method were as much as 8 per cent too high.

Although the surface tension of water, when measured with the ring supplied with the tensiometer, appears to be correct, this circumstance is entirely due to the chance cancellation of two equally large but

opposite errors; and it is evident from the data given in Figs. 1 and 2 that the use of the same ring would not give the correct surface tension value for water at another temperature, or that the correct value would be obtained for any other liquid or solution at the same temperature at which the value for water was by chance found to be correct.

When absolute values are not required, the ring method could be used to note the relative change of surface tension with time as it takes place in the case of many semicolloidal solutions, and then only

TABLE I.

Data illustrating the difficulty of obtaining consistent, reproducible results for a certain time period, and of noting the nature of the change of surface tension with time in the case of gelatin solutions, by the use of the ring method.

Time elapsed after formation of interface	Surface tension in dynes per cm.				
	Solution *A*			Solution *B*	
	Series 1	Series 2	Series 3	Series 1	Series 2
min.					
0.25	62.57	62 43	61.83	60.87	63.87
0.50	62.23	62 17	56.99	61.08	63.25
1	62.30	61.63	58.49	61.42	62.85
2	62 71	61.49	58.56	60.08	62.30
4	62.25	61.27	58.90	61.83	61.63
8	62.23	61.49	58.09	61 21	60.87

Solutions *A* and *B* are both 1 per cent solutions of isoelectric gelatin. While making the surface tension measurements the temperature of these solutions was kept at 35° by surrounding the dish containing the solutions with a jacket through which water from a well regulated water bath was circulated continuously.

when the phenomenon repeats itself in the same manner with successive samples of the same solution (*i.e.* when the rate of change is the same.)

It frequently happens however that the change of surface tension with time does not proceed in the same manner with different samples of the same solution. Such occasional irregularities were illustrated in the case of solutions of gelatin (6) and of soap (7), when the surface tension of these was measured by the capillary rise method. By the use of this method, the exact nature of the change could be followed

because all of the measurements which were made at chosen intervals were made with the same interface. If each measurement had been made with a different sample of the solution, the change could not have been followed because of the possibility that the change with time might have been somewhat different in the case of each interface. Mr. Heinrich, working in this laboratory, has encountered this difficulty in attempting to note the change of surface tension with time in the

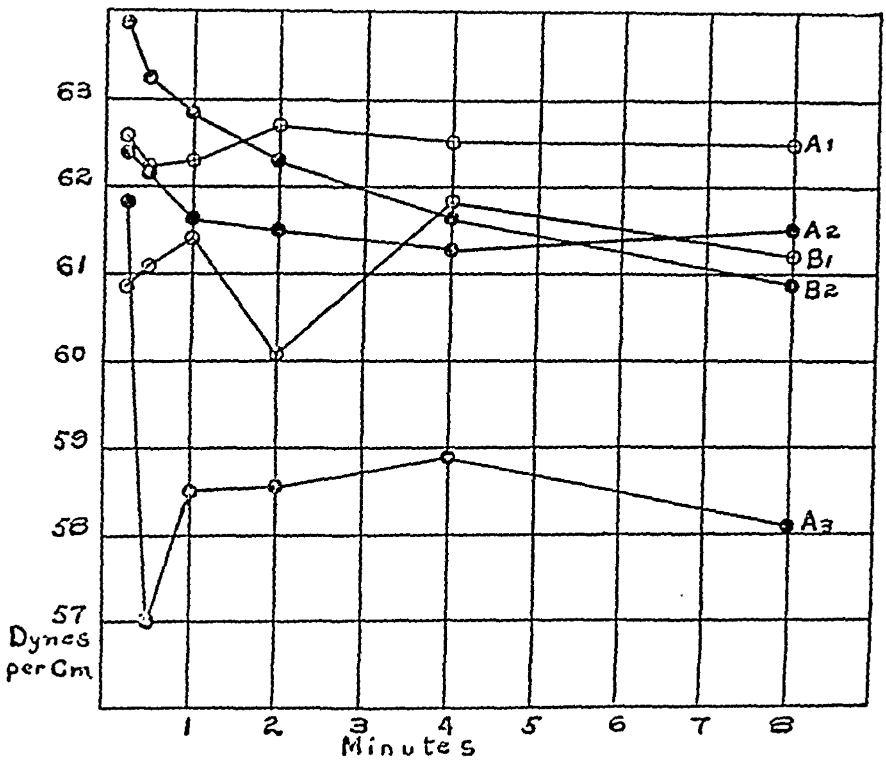

FIG. 3. Illustrates the irregularities of the data obtained with 1 per cent solutions of isoelectric gelatin by the ring method.

case of solutions of isoelectric gelatin. By the ring method it could not be definitely shown that a decrease in surface tension was later followed by an increase, as was found in some cases by the use of the capillary rise method, because the course of the change that takes place with one interface cannot be followed by the ring method. The nature of the results of Table I indicates, however, that the same irregularities were shown with the use of the ring method as when the

capillary rise method was used, and further indicates the impossibility of invariably obtaining consistent results with a solution of this kind.

If it were assumed, on the other hand, that there was a method by which the surface tension of solutions of semicolloids could be accurately measured so as to obtain absolute values, there still remains the difficulty of interpreting the data. In the case of protein solutions, unless they are diluted tremendously, a state of equilibrium that can be considered reliable is seldom reached. In the second place, the rate of change of surface tension with time differs in the case of similar solutions and frequently in the case of different samples of the same solution, and sometimes the rate changes after a time in the case of a specific interface. It is naturally impossible to compare data relative to an equilibrium condition that is never reached, or relative to a definite period of time when the rate of change in the cases to be compared is different.

It has been found that in some instances the change of surface tension with time proceeds in a regular manner and can be expressed by an equation, $\sigma = a\,t^n$, where σ equals the surface tension in dynes per cm., t the time elapsed after the formation of an interface, and a and n are constants. Attention should be called to the fact that the equation $\sigma = \sigma_0 e^{-kt^{\frac{1}{2}}}$, still given in various text-books, is incorrect, and does not fit the data to which it is applied—as was pointed out when the above more correct equation (8) was given by the author several years ago.

SUMMARY AND CONCLUSIONS.

1. Data are given to show that surface tension values obtained by the ring method cannot be considered reliable when absolute values are desired.

2. Data are given to show that, in the case of solutions of semicolloids, surface tension values obtained for a definite period of time following the formation of a new interface cannot always be consistently reproduced by the ring method.

3. It is shown that in the case of solutions of semicolloids, equilibrium values are not readily obtained and should generally not be assumed to have been reached after any definite period of time.

4. The general difficulty of interpreting surface tension values obtained in the case of solutions of semicolloids, by any method, is emphasized.

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ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume is appearing simultaneously with Volumes IX and X. Number 1 of this volume will contain a biography of Dr. Loeb. It is to appear after Number 6, and the page numbers will be roman numerals. The publication of this volume began September 18, 1925.

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THE JOURNAL OF GENERAL PHYSIOLOGY

AVENUE A AND 66TH STREET, NEW YORK, N. Y.

THE DECOMPOSITION OF HYDROGEN PEROXIDE BY LIVER CATALASE.

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(Accepted for publication, September 23, 1927.)

The decomposition of hydrogen peroxide by catalase has been the subject of many investigations, notably those of Senter (1), Sörensen (2), Michaelis and Pechstein (3), Yamasaki (4) and Morgulis and his coworkers (5–7). Different results have been found by these and other authors, the difference lying in the various conditions of temperature, peroxide concentration, etc., employed. The two most comprehensive studies of this reaction are those of Yamasaki and Morgulis. Yamasaki (4) studied the reaction in dilute solutions of hydrogen peroxide (0.02 to 0.1 N) using catalase extracted from vegetable sources, and found that the enzyme was inactivated during the reaction, the rate of decomposition of hydrogen peroxide and the rate of inactivation of catalase bearing a constant ratio to one another. He embodied this relationship in an integrated expression which fits his results well. This equation will be mentioned later. His work is limited, however, since he worked with unbuffered hydrogen peroxide solutions, no knowledge of the effect of pH on the decomposition, therefore, being available from his data. Sörensen (2) has shown that pH has a marked effect on the decomposition of hydrogen peroxide by blood catalase. The work of Morgulis and his coworkers (5, 6) which was performed in more concentrated solutions of hydrogen peroxide (above 0.2 N) although covering a wide range of conditions of temperature, pH and peroxide concentration loses much of its value as a physicochemical study owing to the faulty temperature control. This criticism does not apply to their last paper, Morgulis and Beber (7). In spite of the amount of work already done upon this enzyme it was thought of interest to reinvestigate certain aspects of the com-

plex problem which it presents with the object of confirming and of extending (in certain directions) the results and conclusions drawn by previous workers.

Theoretical.

It will be seen from the results quoted later that the following conclusions, in general agreement with those of previous authors, can be drawn.

A. The velocity of decomposition of hydrogen peroxide is proportional to the concentration of catalase.

B. In dilute solutions of peroxide (up to 0.1 N) the velocity of reaction is proportional to the concentration of hydrogen peroxide, but as the peroxide concentration is increased the velocity becomes independent of the hydrogen peroxide concentration. In still more concentrated solutions of peroxide a depression of the activity of the enzyme occurs.

C. It has been generally found by previous authors and confirmed by the writer, that the reaction gives falling unimolecular constants, ascribed by Yamasaki to the fact that the enzyme is also inactivated during the decomposition of hydrogen peroxide. For dilute solutions of hydrogen peroxide, the velocity of reaction is proportional to both the concentrations of hydrogen peroxide and catalase, hence

$$\frac{-dS}{dt} = k_1 \cdot E \cdot S \tag{1}$$

where S is the concentration of H_2O_2, determined in the present case by titration with standard $KMnO_4$ solution, E is the concentration of catalase and k_1 is the velocity constant.

The value of E, the concentration of catalase, cannot be measured directly. From equation (1) we see that

$$k_1 \cdot E = \frac{1}{S} \cdot \frac{dS}{dt} \tag{2}$$

The results of a typical experiment are given in Table I. The values of dS/dt and dE/dt were obtained by drawing tangents to the curves obtained by plotting the values of S and $k_1.E$ respectively against time. As will be seen from Table I, the ratio of the two velocities is

sensibly constant. Although in all the experiments the constancy is not so good as the above, yet over the greater part of the reaction a fair constancy is obtained. It is seen therefore that in the decomposition of hydrogen peroxide by catalase two simultaneous reactions are going on, namely the catalytic decomposition of the peroxide and the induced inactivation of catalase. This process is called the "induced inactivation" to distinguish it from the so called spontaneous inactivation of the enzyme which only proceeds when the temperature is raised. The velocities of the two processes, namely induced

TABLE I.

1 cc. of a catalase solution (strength 0.09) was mixed with 50 cc. of hydrogen peroxide solution of a concentration of 0.056 N. pH = 6.8, temperature = 15°C.

Time	C H_2O_2	dS/dt	$\frac{1}{S}\cdot\frac{dS}{dt} = k_1 \cdot E$	dE/dt	$\frac{dE}{dt}/\frac{dS}{dt}$
min.	*mols/liter*				
0	0.05680	.00227	0.04	.0038	1.7
2	0.05304	.0018	0.034	.00298	1.66
5	0.04733	.00113	0 024	.00187	1.65
11	0.04242	.00067	0.0158	.00106	1.6
15	0.03975	.00054	0.0135	.00064	1 2
20	0 03781	.00042	0.0112		
25	0.03569	.00030	0.0085		
30	0.03432				

inactivation of the enzyme and decomposition of hydrogen peroxide, are connected by the relation

$$dE/dt : dS/dt = \text{a constant} \tag{3}$$

The value of this proportionality constant varies with catalases from different sources. It also varies with temperature, pH and the previous history and treatment of the enzyme solution. The value for a solution of catalase did not alter sensibly while it was in use, and as the experiments in each section of the present paper were performed with the same solution of catalase, the dependence of the constant upon the above mentioned factors will not affect the value of the results.

Equation (3) is the relation of Yamasaki, and embodied by him in an integrated equation. We have yet to enquire into the significance of equation (3). It states that the velocity of induced inactivation of catalase bears a constant relationship to the velocity of decomposition of hydrogen peroxide. This may mean that the enzyme is inactivated during the decomposition of the peroxide molecule, or it may be inactivated as a sequel to the reaction, the destruction of the enzyme being caused by some factor the magnitude of which is proportional to the velocity of decomposition of hydrogen peroxide. Various possibilities arise for consideration.

1. The inactivation of the enzyme may be caused by the *substrate*, hydrogen peroxide. This is implicitly assumed by Yamasaki and Morgulis. Although in his conclusions he states that either the substrate or the products may be responsible for the inactivation, Yamaski implicitly assumes that the substrate is responsible by writing the kinetic equations in the form:

$$-dS/dt = k \cdot E \cdot S \text{ and}$$
$$-dE/dt = k'E \cdot S$$

where k and k' are the respective velocity constants of the two reactions. Morgulis also assumes that the destruction of catalase in the more concentrated solutions of hydrogen peroxide which he employs consists in the oxidation of catalase by the excess peroxide. It is generally found, as instanced later, that in concentrated solutions the substrate exerts a depressor effect on the activity of enzymes. The process of induced inactivation however is going on in all concentrations, though sometimes masked when the enzyme is in great excess.

2. One factor which is proportional to the velocity of decomposition of hydrogen peroxide is the heat effect. We have called the inactivation of catalase in presence of its substrate "induced inactivation" to distinguish it from the so called spontaneous inactivation which takes place when solutions of catalase (or any other enzyme) are heated alone. Since the decomposition of hydrogen peroxide by catalase takes place at the enzyme surface a possibility of localized heating of the enzyme presents itself, in which case the induced inactivation and the spontaneous inactivation would be identical. Mellor (8) states "The decomposition of hydrogen peroxide into water and molecular

oxygen gives out sufficient heat, if confined to the components of the reaction to raise their temperature 1000°C." If the induced inactivation of catalase was the same as the spontaneous inactivation, in this case brought about by the localisation of the heat of reaction, we should expect them to possess the same critical increment. As will be seen later, the induced inactivation of catalase in dilute hydrogen peroxide solutions possesses a very small critical increment, whereas the critical increment of the spontaneous inactivation of enzymes is usually of the order of 50,000 to 100,000 calories; so that we may conclude that in dilute solutions of hydrogen peroxide, at least, the induced inactivation of the enzyme is not due to local heating.

3. Another possibility which would lead to equation (3) is the inactivation of the enzyme by the products of the reaction. The experiments of Waentig and Steche (9) show that this mode of inactivation is not produced by the molecular oxygen in solution. The other factor, and in the writer's opinion the most probable explanation, is that the induced inactivation is caused by the oxygen, while still in the activated state following its production from the peroxide at the enzyme surface. In this case, we should have

$$- dE/dt = - k_2 \frac{dS}{dt}$$

where k_2 is the velocity constant of induced inactivation.

On this latter basis we have

$$- dS/dt = k_1 \cdot E \cdot S \tag{1}$$

$$- dE/dt = - k_2 \cdot \frac{dS}{dt} \tag{3a}$$

whence $E = k_2 \cdot (S + A)$ where $A = (E_0/k_2 - S_0)$. E_0 is the initial concentration of enzyme and S_0 the initial concentration of substrate.

Substituting this value for E in (1) and integrating we obtain

$$k_3 = k_1 \cdot k_2 = \frac{1}{A \cdot t}\left(\log_e \frac{S_0}{S} - \log_e \frac{(S_0 + A)}{(S + A)}\right) \tag{4}$$

This differs from the equation of Yamasaki in that on the present writer's interpretation the final constant k_3 is the product of the two

velocity constants k_1 and k_2 (k_1 referring to the decomposition of hydrogen peroxide and k_2 to the induced inactivation of catalase) whereas the final constant obtained by Yamasaki and denoted by him, k', is the single constant of the inactivation process alone. This difference lies in the different significance ascribed to equation (3). There is no direct method of comparing the validity of these two interpretations of the relationship $dE/dt : dS/dt$ = a constant. In general, with other enzymes, it is found that the products of reaction are the potent inactivators of the enzyme, the substrate having no such action in dilute solution. In the case of catalase, it is impossible to vary the concentration of substrate and product of reaction independently. Indirect evidence in favour of the writer's point of view may be obtained by a comparison of the behaviour of k_3 with k_2, the constant of

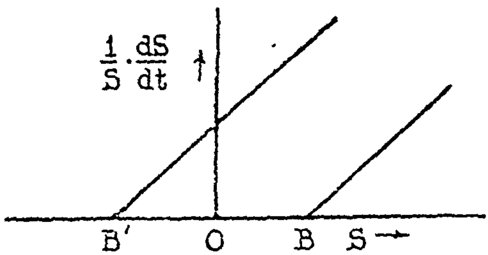

FIG. 1

inactivation alone. Thus whereas k_2 passes through a minimum at pH 7.0 (see Tables V and VI) k_3 shows no such regular behaviour.

Equation (4) has been used by the writer to calculate the results given in Section II of the present paper. The apparent order of the reaction alters with the value of the integration constant, A, as pointed out by Yamasaki: (*a*) If $A = 0$, the reaction gives a bimolecular constant. (*b*) If A is positive and E_0 is much greater than $k_2.S_0$ the reaction will give a unimolecular constant. These are the conditions chosen by the majority of workers. (*c*) If A is negative, the peroxide is in excess, and the enzyme will be inactivated before all the hydrogen peroxide has been decomposed. All these conditions have been realised in the course of this work.

The methods employed to calculate the values of A given later in the tables are the same as those of Yamasaki, namely:

A. Graphical Method.—The value of $\frac{1}{S}\cdot\frac{dS}{dt}$ obtained from the concentration time curve for the experiments is plotted against the concentration of hydrogen peroxide, when an approximately straight line is obtained. The value of A is given by the intercept OB' when A is positive, and by OB when A is negative (*cf.* Fig. 1.)

B. Calculation.—If S_0, S_1, S_2, S_4 are the concentrations of hydrogen peroxide at times 0, t, $2t$, $4t$ and $A_{1,2}$ is the value of A obtained from S_0, S_1, S_2, then

$$A_{1,2} = \frac{2 \cdot S_0 \cdot S_1 \cdot S_2 - S_1^2\,(S_0 + S_2)}{S_1^2 - S_0 \cdot S_2}$$

Similar values of A_1 namely $A_{2,4}$ etc. are obtained in an analagous manner.

The mean result obtained by Methods A and B has been employed throughout.

Calculation of k_1 and k_2.—Substitution of the value of A obtained by the above methods in equation (4) gives the value of $k_3 = k_1.k_2$. Now $A = (E_0/k_2 - S_0)$, and hence $k_3\,(S_0 + A) = k_1.E_0$. The value of $k_1.E_0$ is also obtained by dividing the initial velocity, obtained graphically, by the initial concentration of hydrogen peroxide. This is used as a check on the value of $k_1.E_0$ obtained *via* equation (4). The value of $k_1.E_0$ obtained by equation (4) will be referred to later (*cf.* Tables IV, IV*a*, IV*b*) as $k_1.E_0$ (calculated); the value of $k_1.E_0$ obtained by dividing the initial velocity by the initial concentration of hydrogen peroxide will be referred to as k_1,E_0 (observed).

Definition of Catalase Unit.—To find the value of k_1, we must fix some arbitrary unit for E_0, the initial enzyme concentration, since the molecular unit of an enzyme is not known. The only way of determining the enzyme concentration is to fix conditions of pH and temperature, and define the unit in terms of the velocity constant given. *In this investigation the unit of catalase is chosen as that amount which in a solution of pH 6.8 (obtained with Clark and Lubs' $K\,H_2\,PO_4$ — $NaOH$ mixture) at 20°C. gives a velocity constant (k_1) of unity,* calculated

from equation (4), time being expressed in minutes. It is found by experiment that at 20°C. and at pH 6.8, the value of $k_1.E_0$ obtained for the standard solution of enzyme preparation used in this work is 0.4760. Hence all observed values of k_1,E_0 (obtained with this catalase) must be divided by 0.4760 to reduce them to the unit defined above. This has been done in all values quoted in the present paper.

EXPERIMENTAL.

The catalase used in these experiments was prepared by the method employed by Batelli and Stern (10) and Dixon (11). The brown powder so obtained was stored in a dark bottle. In making solutions for experiments, a weighed amount of the enzyme was ground carefully with small quantities of CO_2-free water, which had been well boiled and cooled to below 10°C., and made up to 100 cc., any insoluble matter being removed by filtration. The strength of the enzyme solution was determined by performing an experiment under the standard conditions mentioned above.

The solutions of hydrogen peroxide were prepared by diluting Merck's perhydrol to the required dilution with distilled water and solutions of buffer salts. The buffer mixtures used were those described by Clark (12) and Kolthoff (13), the latter for the wider pH range. The pH of the solution was measured colorimetrically by means of indicators.[1]

The experiments were performed in a thermostat accurately controlled to within 0.05°C. of the required temperature. The solutions were stirred mechanically by a glass stirrer doing 50 revolutions per minute. The decomposition was followed by titration of samples withdrawn at regular intervals into $\frac{1}{2}$ cc. of strong sulfuric acid (which stopped the reaction immediately), with standard $KMnO_4$ solution. Blank experiments were performed to guard against errors due to the spontaneous decomposition of hydrogen peroxide. It was found that no such decomposition took place during the time of the experiments described in the following sections.

I.

The Effect of Catalase Concentration.

Experiments were performed both in concentrated (0.2 N) and dilute (0.057 N) solutions of hydrogen peroxide, varying the catalase concentration. It will be seen that the initial velocity is proportional to the amount of catalase taken. In the dilute solution used in the

[1] The criticism brought forward by McBain, Dubois and Hay (14) is not applicable here, as the comparator solutions were made up with the same salts as those used in the preparation of the buffered solutions.

experiments summarised in the last section of Table II, the activity of the enzyme is given by $k_1.E_0 = \frac{1}{S_0}.\frac{ds}{dt_t} = 0$ (*cf.* equation (2)), while in the more concentrated solutions of the first two sections of Table II the velocity is independent of the concentration of hydrogen peroxide, as seen below. In this latter case, the true activity of

TABLE II.

Stock catalase solution, strength 1.1 (units defined above). pH = 6.8, concentration of H_2O_2 = 0.22 N, temperature = 20°C.

Relative amount of catalase	Initial velocity	Relative activity
	mols H_2O_2/min.	
4	0.06	4.02
2	0.03	2.01
1	0.0143	1.0

Stock catalase solution, strength 5.4. pH = 6.8, concentration of H_2O_2 = 0.22 N, temperature 20°C.

1 0	0.27	1.0
1.5	0.428	1.59
3.0	0.857	3 17
5.0	1.429	5.29

Stock catalase solution, strength 0.084. pH = 6.8, concentration of H_2O_2 = 0.057 N, temperature 20°C.

Relative amount of catalase	Activity given by $k_1 \cdot E_0$	Relative activity
1.0	0.04	1.0
1.5	0.063	1.57
2.0	0.078	1.95
2.5	0.10	2.5

the enzyme would be given by the initial velocity divided by some constant representing the concentration of hydrogen peroxide at which the velocity becomes independent of it. As is seen, the activity of catalase is proportional to the amount of catalase taken. This is the general fact found for all enzymes.

II.

The Effect of Hydrogen Peroxide Concentration on the Activity of Liver Catalase.

It is generally found for enzyme reactions that whereas the velocity of decomposition is directly proportional to the concentration of enzyme, no such proportionality exists between the rate of reaction and the substrate concentration except in very dilute solution. Senter (1) showed that while the rate was proportional to the concentration of hydrogen peroxide in dilute solution, in solutions more concentrated than 0.1 N the velocity becomes independent of peroxide concentration. To examine this behaviour of liver catalase with varying concentrations of hydrogen peroxide more fully, experiments

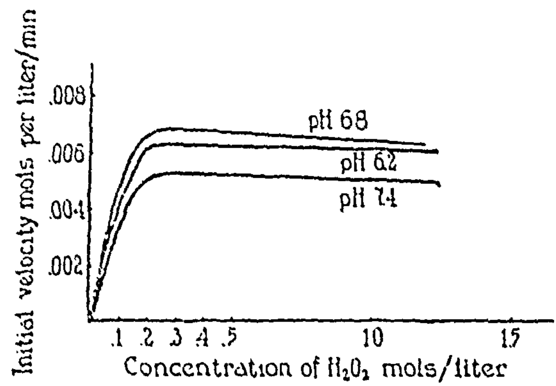

FIG. 2. Variation of initial velocity with H_2O_2 concentration; temperature 15°C.

were conducted over a large range of peroxide concentrations. The experiments were carried out at 15° and 20°C. and at pH 6.2, 6.8 and 7.4 (the optimum activity of catalase being shown by Morgulis and others and confirmed by the writer to be at pH = 6.8). The decomposition was followed for about 10 minutes, the concentration being determined by titration every minute. The values thus obtained were plotted against time and the initial tangent drawn carefully, thus giving the initial velocity. The strength of the catalase solution used in these experiments was 0.18, the strength being calcu-

lated in terms of the unit already defined. The experimental results are plotted in Figs. 2 and 3. It is seen that only for a small range of hydrogen peroxide concentrations does the velocity of decomposition vary in direct proportion to concentration. For pH = 6.8 at 20°C. the relationship has been studied in greater detail. The results for

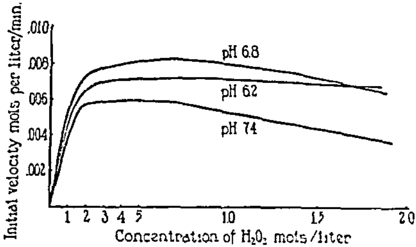

FIG. 3. Variation of initial velocity with H_2O_2 concentration; temperature 20°C.

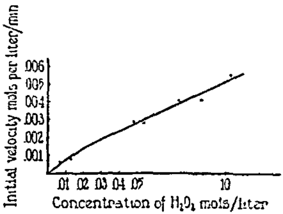

FIG. 3a. Variation of initial velocity with H_2O_2 concentration for low concentration values; pH 6.8, temperature 20°C.

the more dilute solutions of peroxide are given in Fig. 3a. It will be seen that at very low concentrations of hydrogen peroxide the velocity increases more rapidly than is the case for the next portion of the curve which is characterised by a linear relation. As shown in Fig. 3, at still higher concentrations the velocity varies relatively little with

peroxide concentration. In this higher range of concentration, the velocity passes through a flat maximum in the neighbourhood of 0.5 N solutions of hydrogen peroxide. Evans (15) found the maximum to lie between 0.5 and 1.0 N solutions. Morgulis (5) places the maximum activity of catalase at 0.56 N. Curves similar to the first portion of the curves given above (*i.e.* prior to the maximum) have been obtained with other enzymes, with lactase by Armstrong (16), with invertase by Hudson (17) and Nelson and Bloomfield (18), and with malt amylase by Edie (19). These curves are similar to adsorption isotherms in shape. The results of Fig. 3*a* obey a Freundlich isotherm $v - k.C_{H_2O_2}^{0.87}$ which is of the same order as that found by MacInnes (20) for the decomposition of hydrogen peroxide by colloidal platinum. Further support to the view that the decomposition of hydrogen peroxide by catalase involves a process of adsorption is

TABLE III.

Concentration of H_2O_2	$k_{uni.}$	Concentration of H_2O_2	$k_{uni.}$
mols/liter		*mols/liter*	
0.08575	0.0262	0.008573	0.065
0.05257	0.0413	0.006636	0.0686
0.0174	0.064	0.004458	0.0684
0.01256	0.0668		

afforded by the data of Table III. Unimolecular constants have been calculated from the experiments performed in very dilute solutions of peroxide. As will be seen, the values of k_{uni} rise as the dilution of peroxide is increased, reaching a limiting value of 0.066 to 0.068. This behaviour is usually associated with adsorption processes.

Above 0.5 N, the velocity of decomposition commences to decrease with increasing concentrations of hydrogen peroxide. Similar behaviour has been observed in the case of other enzymes. Van Slyke and Cullen (21) find that solutions of urea more concentrated than 10 per cent depress the action of urease, and attribute this depressor action to the high osmotic pressure of the concentrated solutions of urea. The same behaviour is also found for invertase by Nelson and Larson (22). They attribute this behaviour with invertase to de-

creased adsorption of sucrose in concentrated solutions, basing their conclusion on the similarity of their curve to that obtained by von Schmidt-Walter (23) for the adsorption of acetic acid from aqueous solutions by charcoal. Morgulis, Beber and Rabkin (6) attribute the decrease in activity of catalase (as measured by the total amount of hydrogen peroxide which the catalase will liberate in infinite time) with increasing hydrogen peroxide concentration to be due to an oxidation of the enzyme by the excess peroxide. As will be seen from the results of Section IV, Table XII, the activity of the enzyme in concentrated solutions of hydrogen peroxide, as measured by the total amount of decomposition does not follow the same relation as the activity as measured by the initial velocity. Whereas the total amount of decomposition decreases with increased peroxide concentrations at all pH values, being slightly more pronounced on the alkaline side, the initial velocity decreases much more rapidly at pH 7.4, than at pH 6.8 or 6.2, where the velocity remains practically stationary. It is difficult to decide here whether the effect of the high concentrations of peroxide is due to a decrease in the activity of the enzyme surface as a catalyst or to a decrease in the adsorbed concentration at the surface. Both seem to be so intimately connected. This retardation or decrease in activity is accentuated in more alkaline solutions.

III.

The Decomposition by Liver Catalase of Hydrogen Peroxide in Dilute Solutions.

In this region of hydrogen peroxide concentration two simultaneous reactions occur, the enzyme being inactivated (induced inactivation) as the peroxide is catalytically decomposed. The kinetics of the whole process is represented by the expression already deduced, namely

$$k_3 = k_1 \cdot k_2 = \frac{1}{A \cdot t}\left(\log_e \frac{S_0}{S} - \log_e \frac{(S_0 + A)}{(S + A)}\right) \qquad (4)$$

It is of interest to obtain some information as to the variation of the rates of these two processes with the hydrogen ion concentration. The data of Bodansky (24) indicate that two simultaneous reactions affected in a different manner by pH are going on in the system. To

TABLE IV.

pH 4.0, temperature 15°C.

Time	Concentration of H_2O_2	k_3
min.	*mols/liter*	
0	0.05121	—
1	0.04991	(1.05)
2	0.04763	(1.51)
3	0.04660	1.38
4	0.04557	1.31
5	0.04402	1.42
7	0.04181	1.45
10	0.03961	1.38
12	0.03821	1.38
15	0.03682	1.32
20	0.03416	1.39

$A = -0.026.$ Average $k_3 = 1.38$

For duplicate experiment $k_3 = 1.58$

Mean $k_3 = 1.48$

$k_1 \cdot E_0 \begin{cases} \text{(calculated) } 0.035 \\ \text{(observed) } 0.032 \end{cases}$ Average 0.0335. $k_1 = 0.0604$ $k_2 = 24.50.$

TABLE IV*a*.

pH = 8.0.

Time	Concentration of H_2O_2	k_3
min.	*mols/liter*	
0	0.05015	—
1	0.03179	(3.67)
2	0.02400	3.09
3	0.01716	3.14
4	0.01340	2.97
5	0.00964	3.07
6	0.00810	2.87
7	0.00596	2.95

$A = 0.084.$ Average $k_3 = 3.01$

For duplicate experiment $k_3 = 3.31$

Mean $k_3 = 3.16$

$k_1 \cdot E_0 \begin{cases} \text{(calculated) } 0.404 \\ \text{(observed) } 0.40 \end{cases}$ Average 0.402. $k_1 = 0.721$ $k_2 = 4.38.$

TABLE IV*b*.

pH = 9.0.

Time	Concentration of H_2O_2	k_3
min.	*mols/liter*	
0	0.05115	—
2	0.03387	(4.98)
3	0.03078	4.31
4	0.02790	4.07
5	0 02504	4.01
7	0.02143	3.89
10	0.01693	3.95
12	0.01480	4.00
15	0.01244	4 05
20	0.00950	4.28
25	0.00826	4.06
30	0.00604	3.82

A = zero. Average k_3 = 4.04

For duplicate experiment k_3 = 4.44

$k_1 \cdot E_0 \begin{cases} \text{(calculated) } 0.21 \\ \text{(observed) } 0.25 \end{cases}$ Average 0.23. $k_1 = 0.414$ $k_2 = 10.24.$

TABLE V.

Temperature 15°C.

pH	k_1	k_2	k_3
4.0	0.0604	24.50	1.48
5.0	0.202	15.01	3.15
5.6	0.590	5.66	3.33
6.0	0.757	5.15	3.90
7.0	0.856	3.00	2.56
8.0	0.721	4.38	3.16
8.4	0.590	4.51	2.66
9.0	0.414	10.24	4.44

TABLE VI.

Temperature 20°C.

pH	k_1	k_2	k_3
4.0	0.0991	22.7	2.25
5.0	0.261	8.0	2.09
6.0	0.870	4.63	4.03
7.0	0.901	4.22	3.80
8.0	0.784	5.04	3.95
8.4	0.420	6 04	2.54
9.0	0.353	10.40	3.67

investigate the variation in the velocities of the two reactions in dilute solutions of hydrogen peroxide, with change in pH, experiments were performed with hydrogen peroxide solutions 0.05 to 0.055 N, and catalase of strength 1.17 at pH values from 4.0 to 9.0, the pH being attained by the use of Kolthoff's buffer mixtures. The experiments were performed at 15° and 20°C. The results of three typical experiments are given in Tables IV, IV*a* and IV*b*.

It will be seen that in Table IV, A happens to be negative because of the pH value chosen. Tables IV*a* and IV*b* are examples where the value of A is positive and zero respectively.

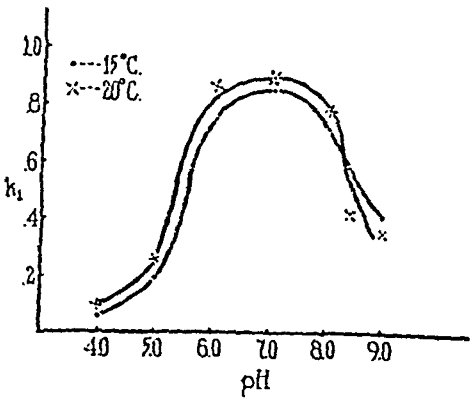

FIG. 4. Variation of k_1 with pH at 15° and 20°C.

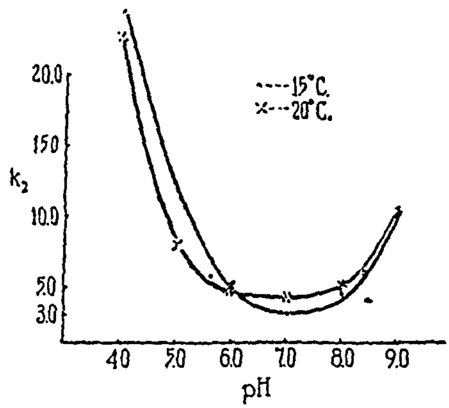

FIG. 5. Variation of k_2 with pH at 15° and 20°C.

The results of the series of experiments, of which Tables IV, IV*a* and IV*b*, are different typical examples, are summarized in Tables V and VI, and plotted in Figs. 4 and 5. Each result is the mean value of two concordant duplicate experiments.

From the results shown in Tables V and VI, it is seen that the activity of catalase given by k_1 rises on both sides to a maximum at about pH 7.0 in agreement with the position of the optimum pH found by others by a different method, while at the same time the value of k_2, the velocity constant of induced inactivation of the enzyme, passes through a minimum at the same pH. It is difficult to state whether the intersection of the curves in Figs. 4 and 5 is a real effect or due to the inherent errors introduced by the rather long and indirect method of calculating the results. It will be seen that except between pH 6.6

and 7.2, there is only a very small difference between the values of k_2 at the two temperatures, *i.e.*, the temperature coefficient or more accurately the critical increment of the induced inactivation of catalase is very small.

In the above cases summarised in Tables V and VI, the initial concentration of hydrogen peroxide, namely 0.05 N, and the catalase concentration were such that equation (4) in its complete form had to be employed. We will now work with a still more dilute solution of H_2O_2, namely 0.03 N, where in the presence of excess of catalase, the

TABLE VII.

Temperature 15°C.

pH	k_1	pH	k_1
6.2	.836	7.0	.884
6.4	.878	7.2	.872
6.6	.896	7.4	.848
6.8	.896		

TABLE VIII.

Temperature 20°C.

pH	k_1	pH	k_1
6.2	.938	7.0	.980
6.4	.991	7 2	.950
6 6	997	7.4	.925
6 8	1.003		

reaction gives a unimolecular velocity constant, namely k_1 (which refers to the catalytic decomposition of hydrogen peroxide), for under these conditions equation (4) reduces to $k_1 = \frac{1}{E_0.t} \cdot \log_e \frac{S_0}{S}$. This latter form is arrived at as follows: $A = \frac{E_0}{k_2} - S_0$. Since E_0 is in excess, E_0 will be much greater than k_2S_0, and A will correspondingly be much greater than S_0 and S so that the term $\log_e \left(\frac{S_0 + A}{S + A}\right)$ of

equation (4) becomes zero and since $A = \frac{E_0}{k_2}$, equation (4) becomes $k_3 = k_1k_2 = \frac{k_2}{E_0.t} \cdot \log_e \frac{S_0}{S}$, that is $k_1 = \frac{1}{E_0.t} \cdot \log_e \frac{S_0}{S}$.

Experiments performed under such conditions are summarised in Tables VII and VIII. The concentration of H_2O_2 solutions was 0.03 N and the catalase solution of strength 0.81. The experiments were performed at 15° and 20°C.

The pH values were obtained with the aid of Clark and Lubs' KH_2PO_4 – NaOH mixtures. From Tables VII and VIII, it will be seen that the optimum pH for catalase is at pH 6.8, which agrees very well with that obtained under the different conditions to which Tables V and VI refer. This is in agreement with the values given by Sörensen (2) and Morgulis (5). At this optimum pH two effects coincide, namely the maximum catalytic activity of catalase and the minimum induced inactivation. It will be seen in the next section that the same behaviour is met with in more concentrated solutions of hydrogen peroxide.[2]

IV.

The Decomposition by Catalase of Hydrogen Peroxide in Concentrated Solutions.

Morgulis and his coworkers (5, 6) have studied the action of catalase on hydrogen peroxide in solutions 0.2 N to 18 N and have noted several interesting features. The results obtained by the writer in general confirm the results of these workers.

In the deduction of equation (4) which fitted the results of the previous section, the fundamental postulate already indicated by Yamasaki and for which further experimental evidence has been offered was that the rates of inactivation of the enzyme and the decomposition of hydrogen peroxide by catalase are connected by the relationship $dE/dt : dS/dt$ = a constant (equation (3)). Furthermore, the induced inactivation was regarded as being due to the product of reac-

[2] Theoretically, the values of k_1 in Tables VII and VIII should be identical with the corresponding values at the same pH and temperature in Tables V and VI. The difference is due to the use of different buffers.

If the amount of hydrogen peroxide decomposed after a time t is x, then it follows that the term $(S + A)$ becomes $(B - x)$ so that equation (4a) becomes

$$k_3' = \frac{1}{t} \cdot \log_e \frac{B}{(B - x)} \tag{4b}$$

which is identical in form with that deduced by Northrop (25).

In Northrop's expression, deduced on the assumption that the enzyme is inactivated in an unimolecular manner with respect to itself, the final constant obtained is the inactivation constant of the enzyme.

TABLE IX.

Catalase solution strength 0.10. Temperature 10°C.

pH	k_1'	k_2	k_3'
5.6	0.0693	0.1095	.00759
5.8	0.0849	0.0862	.00732
6.0	0.0970	0.0757	.00734
6.5	0.1303	0.0593	.00773
6.6	0.1386	0.0541	.00750
6.8	0.1483	0.0539	.00799
7.0	0.1328	0.0575	.00764
7.2	0.1225	0.0618	.00757
7.4	0.1120	0.0652	.00730

Here k_3' is the product k_2, the constant of the inactivation process, k the constant of the catalytic decomposition of hydrogen peroxide and S_l, the limiting concentration of peroxide. The same difference is noted between the expression of the writer and Northrop as was found in the previous case between Yamasaki and the writer, the difference resting on the significance given to the relation $dE/dt : dS/dt$ = a constant. From the experimental results the value of k_3' is readily obtained. As will be seen from (1a) the initial tangent to the concentration time curves for the experiments will give the value of $k_1 \cdot S_l \cdot E_0$. The catalase concentration being known, in terms of the unit previously defined, the value of $k_1 \cdot S_l$ may be calculated, from which $\frac{k_3'}{k_1 S_l}$ gives the value of k_2. The value of the limiting concentra-

tion (S_0) of hydrogen peroxide on catalase is difficult to determine except approximately. It seems to vary with pH between the values 0.09 and 0.14 mols per liter.

Tables IX, X and XI contain a summary of the results obtained with 0.2 to 0.25 N solutions of hydrogen peroxide at different pH and

TABLE X.

Catalase solution strength 0.13. Temperature 15°C.

pH	k_1'	n	k_1'
5.6	0.1536	0.0729	.0125
6.0	0.1515	0.0766	.0116
6.2	0.1495	0.0729	.0132
6.6	0.1670	0.0664	.0111
6.8	0.1696	0.0696	.0114
7.2	0.1464	0.0776	.0114
7.4	0.1351	0.0712	.0129
7.8	0.1379	0.0679	.0115

TABLE XI.

Catalase solution strength 0.10. Temperature 20°C.

pH	k_1'	n	k_1'
5.6	0.1958	0.217	.0139
6.0	0.1616	0.212	.0125
6.2	0.1066	0.212	.0160
6.6	0.1741	0.204	.0164
6.8	0.1858	0.210	.0172
7.2	0.1707	0.167	.0156
7.4	0.1583	0.184	.0202
7.8	0.1135	0.150	.0217

results of these tables are plotted in Figs. 6 and 7. As will be seen, these results show a similar behaviour to those quoted in the previous section for dilute solutions of hydrogen peroxide. The values for k_1' show a maximum at pH 6.8 for the three temperatures. The constant of the inactivation process (k_2) shows a minimum at pH 6.8 for 10° and 15°C., while for 20°C., the value of k_2 is nearly constant, decreasing slightly with increase in pH.

It has already been stated that Morgulis measures the activity of catalase by the total amount of oxygen which it liberates from hydrogen peroxide. A comparison of the behaviour of the initial velocity of decomposition of hydrogen peroxide, used by the writer as a measure

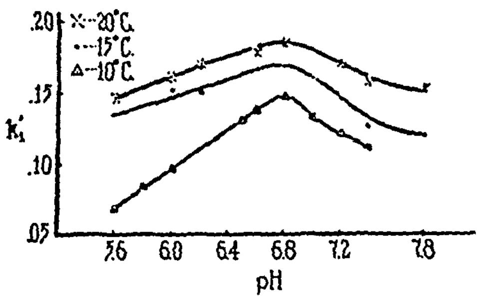

FIG. 6. Variation of k_1 with pH at 10°, 15° and 20°C.

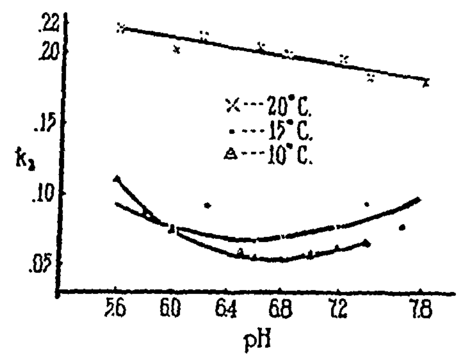

FIG. 7. Variation of k_2 with pH at 10°, 15° and 20°C.

of the activity of catalase ($k_1' \cdot E_0$) and the total amount of decomposition with change in pH is given in Table XII. In each case the relative activity of the enzyme in the least concentrated solution is taken as unity. The same applies to the relative amount of hydrogen peroxide decomposed. The value of k_2 (also relative) is obtained by dividing k_3' by the relative activity of the catalase expressed by the relative initial velocity at that concentration of hydrogen peroxide.

From Table XII, it will be seen that the relative activity ($k_1' \cdot E_0$) and the relative amount of decomposition decrease as the hydrogen peroxide concentration is increased above 0.5 N. Further, whilst the total amount of decomposition decreases with increased hydrogen peroxide concentration at all pH values, the decrease in initial velocity becomes more marked in the more alkaline solutions. On the other hand, the value of k_3' and k_2 at any given pH remain sensibly constant

TABLE XII.

pH 5.8, temperature 20°C.

pH	Concentration of H_2O_2	Relative initial velocity	Relative amount of [illegible]	k_0	Relative k_0
	mol/liter				
	0.1973	1.0	1.0	0.029	0.029
	0.3962	1.375	1.037	0.031	0.023
	0.5898	1.49	0.941	0.036	0.024
	0.7820	1.27	0.878	0.034	0.027
	0.9306	1.25	0.852	.025–.04	.02–.031
	1.2956	1.29	0.762	.02–.042	.015–.03
	1.56	1.22	0.707	.02–.04	.016–.033
6.2	0.1871	1.0	1.0	0.025	0.025
	0.3434	1.284	0.974	0.032	0.024
	0.5600	1.33	0.935	0.033	0.025
	0.7292	1.14	0.803	0.025	0.0245
	0.9135	1.16	0.763	0.027	0.023
	1.3184	1.16	0.647	0.033	0.025
	1.8800	1.0	0.607	.025–.039	.025–.033
6.6	0.1997	1.0	1.0	0.032	0.032
	0.4056	1.0	0.931	0.029	0.029
	0.5861	1.005	0.909	0.025	0.025
	0.7958	1.0	0.879	0.031	0.031
	0.9642	0.926	0.891	0.030	0.032
	1.3532	0.889	0.757	0.025	0.028
	1.9272	0.809	0.628	0.031	0.036
7.0	0.1957	1.0	1.0	0.025	0.025
	0.3400	1.035	0.940	0.027	0.028
	0.5910	1.022	0.946	0.028	0.027
	0.7645	0.914	0.882	0.024	0.026
	0.9076	0.884	0.819	0.022	0.025
	1.2784	0.782	0.773	0.024	0.031
	1.8310	0.742	0.616	0.021	0.027
7.4	0.1993	1.0	1.0	0.021	0.021
	0.3925	1.030	1.003	0.025	0.025
	0.5904	1.045	0.755	0.029	0.028
	0.7744	1.043	0.748	0.022	0.022
	0.9622	0.955	0.669	0.022	0.026
	1.3215	0.852	[illegible]	0.023	0.024
	1.9500	0.663	0.592	0.022	[illegible]

as the concentration of hydrogen peroxide is changed, whereas it was to be expected that since the total amount of hydrogen peroxide decomposed is given by E_0/k_2 (*cf.* equation (5)), k_2 should *increase* in the same proportion as the total amount of decomposition *decreases.* Morgulis, Beber and Rabkin (6) report that their constant corresponding to k_3' remained constant over the range of peroxide concentration to which Table XII refers. It has been noted that in the evaluation of k_3', the second reading is taken as the basis of calculation. This procedure was adopted because of the initial disturbance which takes place. Morgulis, Beber and Rabkin have also noted this initial "outburst" in oxygen evolution, after which the reaction settles down to a steady course. In very concentrated solutions of peroxide, they find that the reaction is entirely confined to this initial stage, lasting a few minutes. It follows that the constant k_3', and hence k_2, refers to this steady course. Before it has been reached, however, the enzyme has been inactivated to a great extent in the concentrated solution of peroxide, as much as 50 to 60 per cent of the enzyme's activity (as measured by the instantaneous velocity of decomposition of hydrogen peroxide) having been lost, so that it is the rate of loss of this remaining 40 to 50 per cent of the activity of the enzyme which is given by k_2, and which remains sensibly constant over the range of hydrogen peroxide concentration considered. The total amount of decomposition however includes both the initial disturbance and the steady course. The discrepancy between the behaviour of k_2 and the total amount of decomposition may be due to the destruction of the enzyme in the initial stage of the reaction.

Quite apart from the question of the oxygen "outburst" effect there is a point to which attention may be drawn in comparing the results of Morgulis and his collaborators with those of the present writer. As already stated, Morgulis measures the activity of the catalase by the total amount of oxygen liberated, the hydrogen peroxide being in excess. The writer on the other hand measures the activity of the catalase by the initial velocity.[3]

[3] Clearly the method of total amount of oxygen liberated would be inapplicable to those conditions wherein the catalase is in excess whilst the initial velocity method would be applicable. Morgulis and his collaborators are concerned however only with relatively concentrated solutions of peroxide and naturally do not suggest that the total oxygen liberated is elsewhere applicable.

TABLE XIII.

Critical Increments Calculated from the Data of Tables V and VI.

pH	E_1.15–20°C.	E_2 15–20°C.
4.0	16,730	—
5.0	8,820	—
6.0	4,700	—
7.0	1,730	11,530
8.0	2,830	4,740
8.4	—	9,870

TABLE XIV.

Critical Increments from the Data of Tables VII and VIII.

pH	E_1.15–20°C.	pH	E_1 15–20°C.
6.2	3,870	7.0	3,460
6.4	4,100	7.2	2,830
6.6	3,630	7.4	2,960
6.8	3,830		

TABLE XV.

Critical Increments Calculated from the Data of Tables IX to XI.

pH	E_1.10–15°C.	E_1.15–20°C.	E_2.10–15°C.	E_2.15–20°C.
5.6	21,600	2,940	—	28,670
6.0	14,540	2,180	383	32,770
6.2	—	4,240	—	27,260
6.6	6,070	2,405	6,680	37,930
6.8	4,380	3,080	8,330	35,670
7.2	5,910	5,090	7,425	31,480
7.4	7,450	4,535	11,650	22,990
7.8	—	5,450	—	23,450

$RT^2 . \dfrac{d \ln k_1}{dT}$. E_2 is the critical increment of induced inactivation and is given by $E_2 = RT^2 . \dfrac{d \ln k_2}{dT}$.

From the data in Tables XIII to XV, it is seen that the critical increment of the catalytic decomposition of hydrogen peroxide by cata-

hydrogen peroxide, which is a maximum at the optimum pH 6.8 to 7.0, and the "induced inactivation" of catalase by the "nascent" oxygen produced by the hydrogen peroxide and still adhering to the catalase surface. This differs from the more generally accepted view, namely that the induced inactivation is due to the H_2O_2 itself. On the basis of the above view, a new interpretation is given to the equation of Yamasaki and the connection between the equations of Yamasaki and of Northrop is pointed out. It is shown that the velocity of induced inactivation is a minimum at the pH which is optimal for the decomposition of hydrogen peroxide.

3. The critical increment of the catalytic decomposition of hydrogen peroxide by catalase is of the order 3000 calories. The critical increment of induced inactivation is low in dilute hydrogen peroxide solutions but increases to a value of 30,000 calories in concentrated solutions of peroxide.

In conclusion, the author wishes to thank Professor W. C. M. Lewis, F. R. S., for his kindly criticism and helpful advice during this investigation. Part of the expense involved was defrayed from the Brunner Mond Research Grant, for which grateful acknowledgment is made.

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1914). The temperature was kept constant by means of a water bath. The apparatus was calibrated and the volumes of the vessels determined by filling with mercury and weighing. The calculation from the pressure of the amount of gas evolved was made according to the method worked out by Warburg (1926)[1] and the amount is expressed as volume per cent.

Hemoglobin.

Barcroft (1914) has developed the method for determining the oxygen in oxyhemoglobin by means of $K_3Fe(CN)_6$. The ferricyanide drives off the loosely combined oxygen and converts the oxyhemoglobin into methemoglobin. The same method has been used by Fox (1926) in his work on chlorocruorin. The data regarding the effect of KCN are not so satisfactory. Kobert (1900) has prepared a cyanide-hemoglobin compound from oxyhemoglobin, and Haurowitz (1924) finds a cyanide derivative of methemoglobin to which he assigns the formula $\mathrm{Hb}\begin{smallmatrix}\diagup \mathrm{OH}\\ \diagdown \mathrm{CN}\end{smallmatrix}$. Abderhalden (1911) cites several other investigators who have found the same or similar compounds. There seems to be no doubt, therefore, that the CN radicle can combine to some extent with hemoglobin.

A few preliminary experiments were made on hemoglobin, principally to test the accuracy of the apparatus. Fresh cow's blood was obtained from the slaughter house, defibrinated, the plasma separated by centrifuging, and the corpuscles laked with distilled water. The clear solution of hemoglobin thus obtained was saturated with oxygen at atmospheric pressure and tested with 10 per cent $K_3Fe(CN)_6$ and 10 per cent KCN in the Barcroft apparatus at 17.0°C. The results are shown in Table I.

From the above it will be seen that as determined by the ferricyanide method cow's blood contains about 22 volumes per cent O_2. Barcroft (1914) has found human blood to contain about 18 to 19 volumes per cent O_2 and other bloods to contain varying amounts of the same order

[1] Warburg has used a somewhat different method from Barcroft for calculating the results. The details may be found in the introductory chapter of his book (Warburg, 1926).

To obtain the blood of *Homarus* an incision was made in the ventral artery at the first abdominal segment and the blood allowed to flow out. With *Maia* a hole was pierced in the back of the shell just over the heart, and the animal quickly inverted over a beaker. The blood pours out with great rapidity and a large quantity may be secured.

The blood of *Homarus* coagulates with considerable rapidity. It was prepared for use here by three methods: (*a*) dilution with an equal quantity of distilled water, (*b*) treatment with sodium oxalate, and

TABLE II.

Effect of KCN on Hemocyanin.

Results of Twelve Experiments Performed at 18°C. and Atmospheric O_2 Tension.

The letters (*a*), (*b*), (*c*) refer to the method by which blood was kept from coagulation (see text).

	Volume per cent O_2, Stedman and Stedman	Volume per cent O_2 with Barcroft apparatus
Homarus	1.22	(*a*) 0.71
		(*b*) 0.78
		1.05
		(*c*) 0.90
Maia	1.68	
	1.29	1.22
		3.07
		2.11
		0.79
		1.25
		1.17
		1.81
		2.70

(*c*) immediate removal by centrifuging of the clotted elements. The latter procedure sufficiently delays but does not ultimately prevent the coagulation of the serum. In all cases the volume per cent of oxygen was calculated on the basis of the quantity of the original blood present.

The blood of *Maia* does not coagulate but the organized elements form a small clot. This clot was removed and the clear blue blood used.

in hemoglobin and hemocyanin. Marrian has plotted the dissociation curve of oxyhemerythrin and has shown that it can be converted by potassium ferricyanide into a compound which he calls methemerythrin analogous to methemoglobin. He was unable, however, to demonstrate the presence of hem or hematoporphyrine and therefore regards hemerythrin as an iron-protein compound similar to hemocyanin which is a copper-protein compound. Hemerythrin would thus be intermediate in its properties between hemoglobin and hemocyanin. In such a case the action of potassium cyanide and potassium ferricyanide should be of interest.

TABLE III.

Hemerythrin.

Two sets of animals were used: (*A*) from Salcombe, (*B*) from the River Yealm.

	Volume per cent O_2 with $K_3Fe(CN)_6$	Volume per cent O_2 with KCN
Hemerythrin from (*A*)	4.75 4.70	2.1
Average	4.72	2.1
Hemerythrin from (*B*)	3.3 2.7	1.70 0.78 1.25
Average	3.0	1.17

Hemerythrin was obtained from a species of *Phascolosoma*, a worm living in tidal mud flats. The posterior end of the animal was cut off and the body fluid squeezed out into a test-tube. Each animal yields several drops. The fluid was then centrifuged, the supernatant liquid drawn off, and the corpuscles (comprising 5 to 10 per cent of the total volume) laked in distilled water. After centrifuging the débris of the cytolized cells a faintly opalescent madder red solution was obtained. This is the oxidized form of hemerythrin.

Table III shows the effect of adding 10 per cent $K_3Fe(CN)_6$ and 10 per cent KCN to a solution of hemerythrin at 16°C. and atmospheric oxygen tension.

It will be observed that both potassium cyanide and potassium

ferricyanide cause hemerythrin to give up oxygen. Hemerythrin thus stands in contrast to hemoglobin and hemocyanin with each of which only one of these substances is effective.

To account for this effect of the cyanide compounds it must be assumed that the hemerythrin in some way combines certain of the characteristics of hemoglobin and hemocyanin. It contains principally iron (at least copper has not yet been shown to be present) which may be united to a protein in a form similar to that in which copper is supposed to exist in hemocyanin. At the same time it is possible to obtain, according to Marrian, a methemerythrin corresponding to methemoglobin. The formation of this met compound would account for the liberation of oxygen under the influence of potassium ferricyanide and the structure of the protein complex might account for the liberation of oxygen and potassium cyanide. In other words the oxygen may be expelled in two very different ways.

A discrepancy will immediately be noticed. The amount of oxygen obtained with potassium cyanide is only about one-half that obtained with potassium ferricyanide. If all the bound oxygen is set free in both cases the amount should always be the same. The question may be solved in the following manner. Marrian observed that hemerythrin is stable only over a small pH range and that the pigment was irreversibly decolorized by alkali. That the pigment is destroyed by alkali or at least radically altered is shown by an experiment with potassium ferricyanide in which the hemerythrin was first decolorized with sodium hydroxide. There was no evolution of oxygen whatever and, therefore, there could be no oxyhemerythrin present. Furthermore, experiments with sodium hydroxide showed that the decolorization itself is not accompanied by any liberation of oxygen. Under ordinary circumstances the reaction with ferricyanide proceeded at least 10 minutes before completion. But with potassium cyanide the liberation of oxygen always ceased in about 1 minute, a fact which indicates either that the reaction is extremely rapid or that for some reason it is strongly inhibited. Since a 10 per cent solution of potassium cyanide is quite strongly alkaline it is reasonable to suppose that one of its effects would be to destroy the hemerythrin exactly as in the case of sodium hydroxide. There would then be two reactions proceeding simultaneously, one which would destroy the pigment and one

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which would liberate oxygen. Only part of the oxygen would ever appear in the free state, that part which was displaced by the cyanide before all the hemerythrin was decomposed in the presence of the alkali. It may then be stated that both cyanide and ferricyanide drive off the oxygen from hemerythrin and that this substance, in this respect, seems to embody some of the properties of both hemoglobin and hemocyanin.

Echinochrome.

MacMunn (1885) described a pigment obtained from various echinoderms which he named echinochrome and which he considered to be a respiratory pigment. This view was upheld by Griffiths (1892) who found it to contain an iron compound, probably a porphyrine.

TABLE IV.

Results of Twelve Experiments Showing Volumes Per Cent O_2. Removed from Echinochrome Extract by $K_3Fe(CN)_6$.

1.24	0.95
1.79	1.19
0.82	0.85
0.77	1.02
0.62	1.51
0.67	0.92

Winterstein (1909) reports that echinochrome does not contain more oxygen than sea water and therefore cannot be a respiratory pigment.

Cannan (1927) has investigated the oxidation-reduction potential of echinochrome. He considers the substance to be an "activator" of oxygen, not a "carrier" and he finds no evidence, using the ordinary methods, of any dissociable compound with oxygen. Nevertheless, reduced echinochrome may be oxidized by atmospheric oxygen or by mild oxidizing agents, the product being the same in both cases. If oxidized echinochrome is a stable compound one would not expect that oxygen could be removed by potassium ferricyanide. But in twelve experiments performed with the Barcroft apparatus on echinochrome a small but definite quantity of oxygen was given off (see Table IV).

The material was taken from a large species of *Echinus* found near Plymouth. The body fluid was removed through a hole in the test

and allowed to clot. The clot was filtered off and extracted with distilled water, giving a clear yellow solution. The clot itself is deep reddish brown. The extract is also at first reddish brown but changes very rapidly to yellow.

With potassium cyanide no positive gas pressure was observable and, therefore, cyanide does not drive off oxygen from echinochrome. It does, however, cause a rapid colour change from yellow to reddish lavender which more slowly alters again into a yellow. Although these changes indicate that cyanide reacts in some way with the pigment they provide no clue to the nature of the changes involved.

The effect of 10 per cent potassium ferricyanide on echinochrome at 16° and at atmospheric oxygen tension is shown in Table IV.

The variation in these experiments is due partly to the fact that there is much more clot in some animals than in others and partly to differences in concentration of the pigment. The clot was usually extracted by about one-tenth as much distilled water as there was fluid in the animal. Therefore, the real values of the oxygen content of the body fluid are about 10 per cent of those given in Table IV, very small values indeed when compared to those of the other pigments. But regardless of how small the amount of oxygen may be, it is clear that there must be some of this element which is united loosely with the pigment. Even though the quantity is no greater than that dissolved in sea water the fact that it may be driven off with ferricyanide is evidence that it is in chemical combination and not in solution, for oxygen cannot be removed from sea water by the addition of ferricyanide.

In view of Cannan's results it is quite surprising that there should be any oxygen evolution at all, for it might reasonably be expected that if the oxidized form of echinochrome is stable the oxygen could not be removed by ferricyanide. The present state of the problem does not warrant the drawing of any conclusions save that the possibility exists that echinochrome, to some extent, may perhaps act as a "carrier" as well as an "activator" of oxygen.

SUMMARY.

The oxygen in hemoglobin is liberated by $K_3Fe(CN)_6$ and not by KCN, that in hemocyanin by KCN and not by $K_3Fe(CN)_6$, that in

hemerythrin by both, and that in echinochrome by $K_3Fe(CN)_6$ and not by KCN. The bearing of these results on the nature of the substances involved is discussed.

I wish to take this opportunity to extend my thanks to Dr. E. G. Allen for his kindness in extending to me the hospitality of the Plymouth Laboratories, and to Mr. C. F. A. Pantin for his interest and many helpful suggestions during the progress of the present investigation.

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TEMPERATURE CHARACTERISTICS FOR FREQUENCY OF RESPIRATORY MOVEMENTS IN YOUNG MAMMALS.

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I.

It has been long known that the young of such mammals as mice, rats, and man have little capacity for thermoregulation at birth and remain in a sense poikilothermic for some time thereafter (Edwards, 1832; Pembrey, 1895; Babák, 1902; Sumner, 1913).

We have made use of this condition in studying the respiratory movements of mice, 1 to 2 days old, by varying the environmental temperatures. The undeveloped homeothermy makes it possible to extend analysis by thermal increments (Crozier, 1924–25, *a;* 1924–25, *b;* Crozier and Stier, 1924–25, *a*) to the activities of mammals, and so to find if controlling processes commonly recognized by this means in the vital activities of poikilothermic animals are also found with intact, uninjured mammals.

II.

For these experiments we employed twenty animals of the 4th to 5th inbred generations from controlled brother-sister mating in a number of comparable lines of piebald mice derived from common stocks which had been previously loosely inbred.[1]

The differences between the internal temperatures of these mice and that of their environment were obtained by the method of thermometry commonly employed for such measurements (*cf. e.g.*, Rogers and Lewis, 1916; Pirsch, 1923). A primary thermopile, consisting of three insulated pairs of No. 40 copper-constantin junctions, was in-

* National Research Council Fellow.

[1] Further studies with more homogeneous material are now in progress; it is hoped that we may obtain findings of value for genetic analysis.

serted into the rectum of the young mouse; the reference thermopile, consisting of three of the same bimetallic junctions joined in series with their corresponding primary junctions, extended 3 cm. into the beaker surrounding the mouse. A difference in temperature between the two thermopiles resulted in a deflection of the galvanometer. By direct calibration of this circuit, each mm. on the galvanometer scale represented a temperature difference of about 0.18°C. The temperature of the chamber containing the mouse was kept constant to at least ±0.05°C. by an outside water thermostat, and by the circulation of air which was first bubbled through water and then passed through 20 feet of coiled glass tubing immersed in the water bath. A standard thermometer reading to 0.01°C., the thermopile leads, and the air line, entered the chamber through a hole in the plate glass window covering the beaker, in such a way that the respiratory movements of the animal were not obscured. The mouse was held on a strip of cork by three strips of adhesive tape. Whether the mouse was held dorsum up or belly up made no apparent difference in the respiratory rates. However, when the abdomen is clearly exposed, movements of the ribs, abdomenal musculature, and especially of the milk-filled stomach, can be more easily seen. It may be stated that without practice there is great difficulty in observing the respiratory movements accurately, since recurrent periods of activity change the muscle tensions and so tend to mask the rhythmic contractions of the body wall. Consequently one of us always observed the respiratory movements, taking with a stop-watch the time for ten inhalations, while the other observer noted the deflection of the galvanometer during the reading.

We secured many detailed observations regarding the characteristics of the galvanometer deflections which occur at constant air temperatures, the increase of the deflection with rise in temperature of the surroundings, and the increasing difference between the internal and the external temperature with advancing age of the animal. But these data are of more particular interest for an account of the development of the thermoregulatory mechanism in mice. It will suffice to note that when the outer temperature is changed the internal temperature alters to a definite level above that of the environment and is then maintained constant at this new level. For 2 day old mice this equilibrium temperature was found to be 0.1°C. hgiher than the external temperature of 16°C., and from 2° to 3° higher at 34°C. The increase in the equilibrium temperature with increase in external tem-

perature may be the resultant effect of the heat produced during the increased "spontaneous" muscular activity[3] and of the rate of cooling which probably follows Newton's law of cooling as found for example in chickens (Mitchell and Haines, 1927).

III.

A number of investigators (*cf.* Crozier, 1924–25, *b* and 1925–26, *b*) have measured the rates or frequencies of vital activities in excised mammalian organs and tissues. The "temperature characteristics" for these data, as calculated (Crozier, 1925–26, *b*) using the Arrhenius-Marcelin-Rice equation, fall into those classes of values typically found for vital processes in general. We also find certain of these modal values of μ when we plot the *log frequency of respiratory movements* against the *reciprocal of the absolute temperature.* The calculations are based on 3600 stop-watch readings with twenty animals in twenty series of "runs." Typical plots are given in accompanying figures.

In obtaining these data we made special effort to take thirty to thirty-five readings at each temperature, because we quickly noted that unusually wide variation appeared in the readings. With such a large number of observations it is then possible to catch the extreme rates, which repeat themselves cyclically, as earlier shown in the case of respiratory movements of grasshoppers (Crozier and Stier, 1924–25, *a*). The plots clearly show this variation, but they also show that throughout the temperature range it is expressed as a constant percentage of the mean, thereby making the latitude of variation of constant width (logarithmically) except where the thermal increment changes abruptly. In spite of the comparatively wide latitude of variation, the means and the extreme variates are fitted by the Arrhenius equation with a precision comparing favorably with that in other cases previously published. It is often overlooked that a primary desideratum for such treatment is many readings at each constant temperature over a sufficient period of time (Crozier and Stier, 1926–27).

It might be expected that since there is such variability in the readings, due to cyclic variations in frequency of breathing and to errors arising from difficulties encountered in observation, the calculated values of the thermal increments might be distributed at ran-

[3] Unpublished experiments (T. J. B. S.), to be considered later.

dom. However, in the accompanying table it is seen that the values of μ closely group themselves in commonly occurring classes (*cf.* Crozier, 1925–26, *b*).

No. of instances	Modal μ	Extreme range
1	8,000	8,450
6	12,000	12,000–12,800
2	18,000	18,100–18,190
4	28,000	28,130–29,000
1	33,000	33,000
3	36,000	36,300–36,600

Each of the values (save the last) falls into a heavily weighted class of μ's as these are obtained from all available data on protoplasmic activities. As with poikilothermic organisms, definite critical temperatures occur above which or below which the effect of temperature change is only very slowly reversible; or at which there are abrupt changes in the values of the μ obtained. These temperatures are at the modal values 34°, 20°, and 15°C., as have been obtained in numerous other instances (*cf.* Crozier, 1925–26, *a*).

No. of instances	Average critical temperatures	Variation
	°C.	°C.
2	15.5	16.7–14.2
6	19.8	21.1–18.7
3	35.2	37.4–32.7

It is obvious that here again, as in earlier instances, it becomes absurd to uncritically average together measurements from different individuals.

IV.

Winterstein (1911) and others have viewed the control of the activity of the medullary respiratory centre as by hydrogen ion. If the frequency of respiratory movements in the mouse were determined by some process catalyzed by H ion, it might be expected that the temperature characteristic for respiration in mice should correspond to

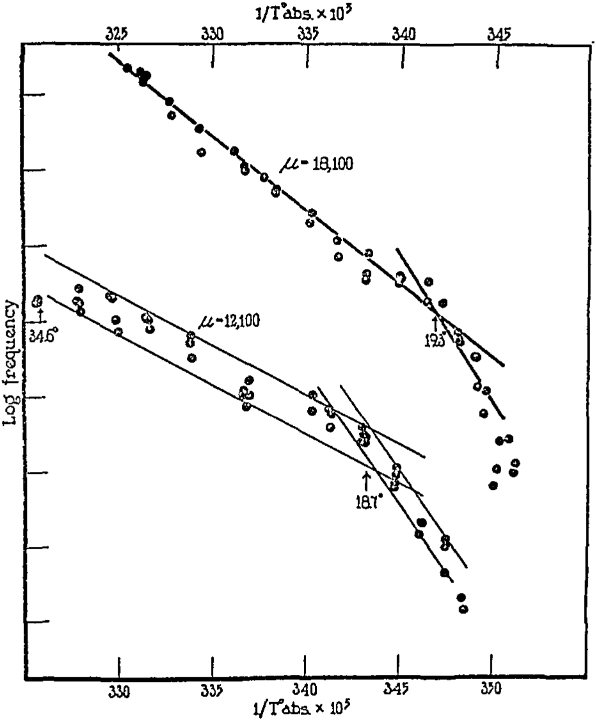

FIG. 1. Upper curve: the critical increment for respiratory movements of mouse No. 18 is 18,100 calories, with an abrupt change of increment at 19.3°C.

Lower curve: the critical increment for respiratory movements of mouse No. 16 is 12,100 calories from 18.7° to 34.6°C., and 33,000 calories from 14.2° to 18.7°C.

The data for mouse No. 18 are plotted against the upper temperature scale, those for No. 16 against the lower temperature scale.

that obtained for known H catalyzed processes (*i.e.*, μ = about 20,000). However, for no example of respiratory movements studied in this laboratory is this value of μ obtained (*e.g.*, Crozier, 1924–25, *b*; Crozier

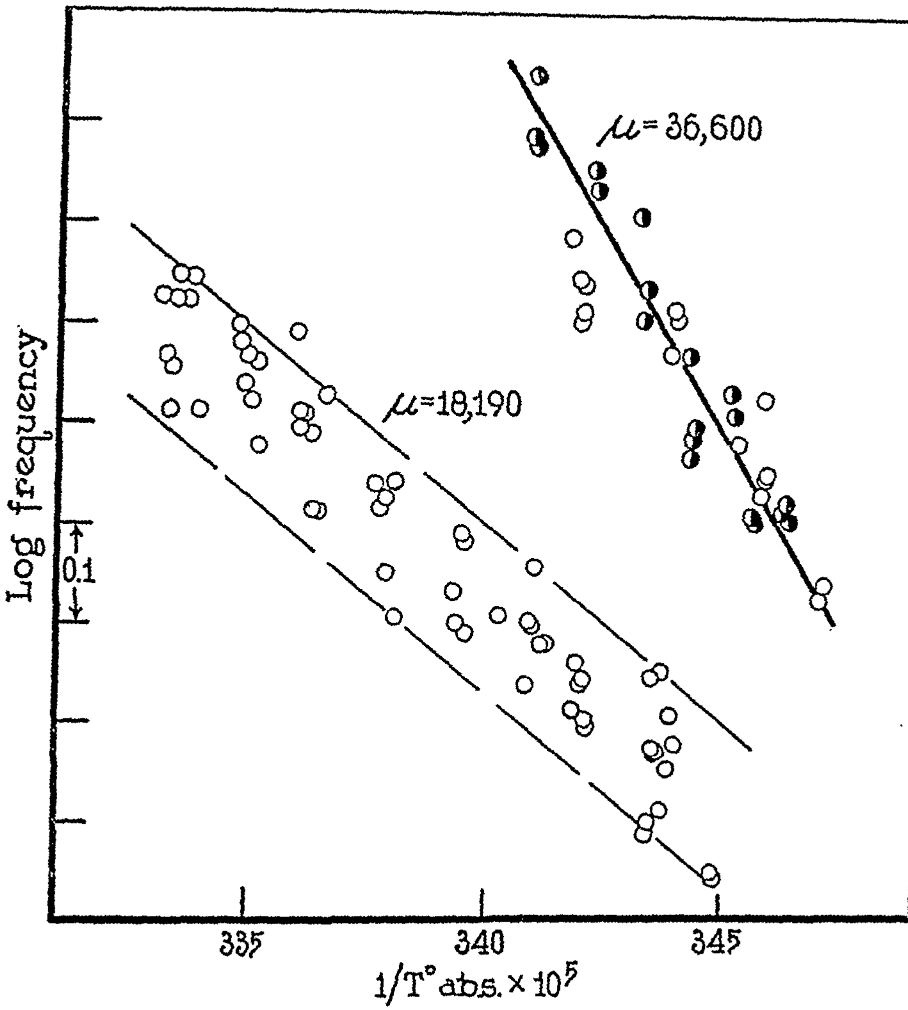

FIG. 2. Upper curve: mass plot of data for mice No. 4 (open circles) and No. 13 (half circles) gives a critical increment of 36,600 calories from 15.1° to 20.5°C.

Lower curve: data for mouse No. 6 give a critical increment of 18,190 calories with no abrupt change of increment.

and Stier, 1924–25, *a*, *b*, *c*; 1925–26). There is thus no direct evidence given for the control of respiration by a simple change in the pH of the fluid bathing the respiratory centre (*cf.* Gesell, 1923; Gesell and Hertz-

man, 1924–25). The effects of varying the O_2 pressure also adds evidence conflicting with a hydrogen ion hormonal control of respiration (Campbell, 1927). As judged from critical increments, in the uninjured young mammal respiratory frequency is controlled by the activation of catalysts similarly found to be the controlling links in some nexus of chemical reactions occurring in tissues of lower vertebrates and invertebrates.

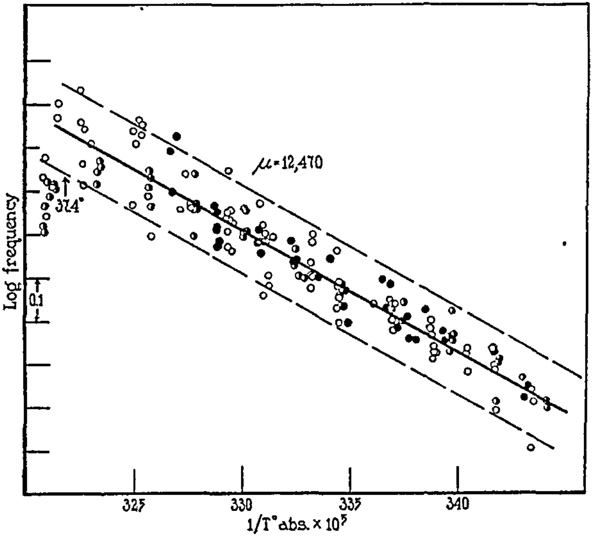

FIG. 3. Mass plot of data for mice: No. 2 (closed circles), No. 3 (open circles), and No. 8 (half circles) gives an average μ of 12,470. The data for mice Nos. 2 and 8 are multiplied by a factor.

The value of 12,000 for μ which we obtained most frequently has been found primarily associated with neuromuscular activities of arthropods (except breathing movements), being obtained for velocity of progression in *Parajulus*, and ants; frequency of chirping in tree crickets; frequency of flashing in fireflies; frequency of heart beat in

Limulus, *Ceriodaphnia*, and *Bombyx* larvæ (Crozier and Stier, 1924–25, *a*). This is the first time that it has been obtained for frequency of respiratory movements, although examples of its occurrence might be cited in (unpublished) experiments by Stier and Crozier with echinoderms.

SUMMARY.

The internal temperature of 2 day old mice deviates by +0.01° to +3.0°C. from the environmental temperature over a range of 24°. The undeveloped temperature control allows rhythmic activities in this mammalian material to be readily subjected to the analysis afforded by temperature characteristics, and thus makes possible some insight into the physicochemical events controlling vital processes in entire uninjured mammals. The thermal increments and critical temperatures obtained point to a similarity in the controlling system of reactions for both homothermic and poikilothermic organisms. For frequency of respiratory movements the increments 12,340 (19.6° to 35.2°C.); 28,340 or 36,500 (15.5° to 19.6°C.) are most frequently found (thirteen cases). Rarely μ = 8,450, 33,000, or 18,340 (two cases). The last increment is either associated with μ = 36,000 below 20°, or extends unbroken throughout the whole range (one case).

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THE PHOSPHATE ION AND HYDROLYSIS BY PANCREATIC LIPASE.

By E. R. DAWSON and B. S. PLATT.
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(Accepted for publication, November 16, 1927.)

In a recent paper Lyon (1926–27) has used data on the activity of lipase in phosphate buffer solutions of different H^+ and PO_4''' ion concentrations to substantiate a general thesis on the effect of the phosphate ion on certain biological processes. He finds that the results of various workers conform to the equation:

$$(\text{Activity of enzyme})(pPO_4)^n = K,$$

where pPO_4 is the expression introduced by Holt, La Mer and Chown (1925) to represent the concentration of the PO_4''' ion, and n and K are constants. Certain results obtained by the writers on the effect of phosphates on the hydrolysis of esters by pancreatic lipase conformed to the equation put forward by Lyon. It is found, however, that other data are not amenable to this mathematical treatment. Some explanation of the discrepancies is given in the present paper.

In the experiments to be described, the lipase preparation was obtained from pig's pancreas by the method used by Hewitt (1927) for obtaining undenatured proteins from serum. In view of the explanations advanced of the action of lipase based on the insolubility of this enzyme, it should be noticed that by this means a preparation is obtained which is mainly soluble in water and which can be filtered several times, even through a hardened filter paper, to yield a clear solution without any appreciable loss in activity.

The Effect on the Activity of Lipase of Changing the PO_4''' Concentration by Varying the pH.

According to Lyon the hyperbolic relationship between pPO_4 and the activity of the lipase holds whether the concentration of the PO_4'''

ion be increased by increase of pH or by increase of the total salt concentration. That this is not the case is obvious from a consideration of the results given in Fig. 1 of a previous paper (Platt and Dawson, 1925), where it is shown that the enzyme exhibits optimal activity at about pH 7.0 when 0.05 M phosphate mixtures are used. Thus keeping the total phosphate concentration constant while decreasing the pPO_4 by increasing the pH, the enzyme activity first rises and then

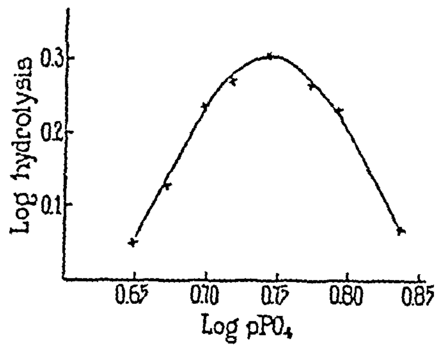

FIG. 1

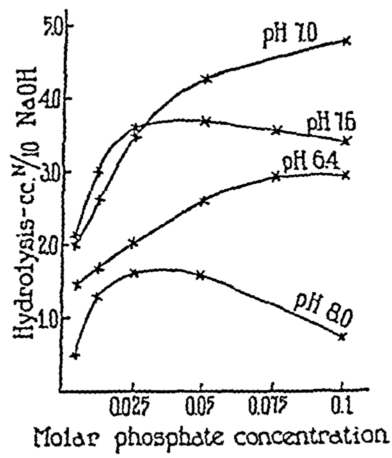

FIG. 2

FIG. 1. Logarithmic plot of the relation between pPO_4 and the amount of hydrolysis of ethyl butyrate. The molar phosphate concentration is the same in all cases, the pPO_4 being altered by adjusting the pH. Experimental details are given in Experiment 2 (Platt and Dawson, 1925).

FIG. 2. The effect of phosphate concentration on hydrolysis of ethyl butyrate by lipase at various hydrogen ion concentrations. The mixtures used contained 10 cc. KH_2PO_4-NaOH buffer mixtures, 2 cc. solution containing 10 mg. of lipase preparation and 0.5 cc. ethyl butyrate. 10 cc. samples were titrated with N/10 NaOH after shaking for 1 hour at 37°C.

falls. These results have been plotted in Fig. 1 below, and show that the activity of the enzyme is optimal at a certain pPO_4.

The Effect on the Activity of Lipase of Varying the PO_4''' Concentration at Constant pH.

If the amount of hydrolysis of ethyl butyrate by pancreatic lipase is determined in solutions of constant H^+ but varying PO_4''' ion con-

centrations, aberrations from the linear relationship occur when the solutions are more alkaline than pH 7.2. This is all the more apparent when the amount of hydrolysis is estimated after a short incubation period, or, in other words, when the values plotted approximate to the initial velocities.

The Effect on Lipase Activity of Treatment with Buffer Solutions of Various PO_4''' and H^+ Ion Concentrations.

The stability of lipase preparations from pig's pancreas varies in buffer solutions of differing H^+ and PO_4''' ion concentration. The

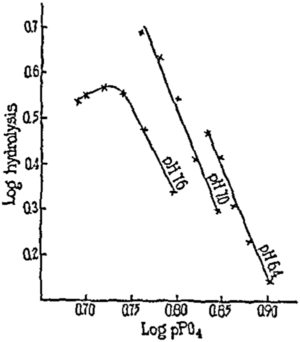

FIG. 3

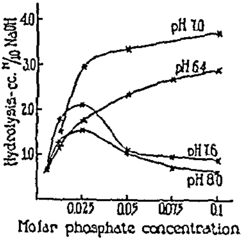

FIG. 4

FIG. 3. The relation between log hydrolysis and log pPO_4, using the values for hydrolysis and PO_4''' ion concentration plotted in Fig. 2.

FIG. 4. The relation between phosphate concentration and amount of hydrolysis of ethyl butyrate in 1 hour at 37°C. by lipase previously treated for 2 hours with phosphate buffer solutions of varying PO_4''' and H^+ ion concentrations. The quantities used were the same as those for the experiment reported in Fig. 2 above.

term "stability" is not to be confused with the sensitivity of the enzyme to changes in concentration of PO_4''' ions. The former is determined by the amount of destruction of the enzyme in the presence of PO_4''' or OH' ions, whilst the sensitivity, measured by the constant n of Lyon's equation, represents the degree to which the hydrolytic activity of a particular preparation is promoted by phosphate ions.

An attempt has been made to separate the effect of PO_4''' and OH' ions on the stability from that on the sensitivity of the enzyme. The influence of the ions on the stability can be intensified by a preliminary treatment with the buffer solutions before the addition of the substrate. The activity of the enzyme after treatment can be determined either by immediate addition of equal amounts of substrate or, by first adjusting all the mixtures to the same pH and phosphate concentration. The former method is simple and has usually been followed since essentially similar results are obtained with both methods. Experiments carried out along these lines showed that solutions more alkaline than pH 7.0 have a marked influence on the stability of the enzyme. Still more marked changes are produced by increasing the phosphate concentration in alkaline solutions.

It should be pointed out that for a complete analysis of the reaction, the effect of ions other than those considered above, as well as the influence of the undissociated salt, should be taken into account.

SUMMARY.

The equation, (activity of enzyme) $(pPO_4)^n = K$, has been investigated and has been shown to have only a limited application to the effect of the phosphate ion on the hydrolytic activity of pancreatic lipase. The deviations observed are ascribed to the effect of certain factors on the stability of the enzyme.

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THE GEOTROPIC REACTION OF RODLESS MICE IN LIGHT AND IN DARKNESS.

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(Accepted for publication, November 21, 1927.)

I.

It has been shown that it is possible to formulate precisely the geotropic orientation and movement of rats (Crozier and Pincus, 1926–27 (3, *c*); Pincus, 1926–27 (4)), mice (Crozier and Oxnard, 1927–28 (7)) and certain invertebrates (Wolf, 1926–27 (5); Crozier and Stier, 1927–28 (6)) through their behavior on an inclined plane. The present paper records preliminary results of a more complete physiological study of the development of sense organs in the rodless mouse employing this method.

II.

The apparatus used was similar to that employed by Crozier and his collaborators, consisting of a plane 2½ feet square overlaid with black screen wire upon which is painted a grid, 5 cm. between intersections. An inverted protractor, to which was attached a weighted thread, measured the angle of inclination of the plane (α). A large celluloid protractor was constructed to measure the angle of orientation upward from the horizontal (θ) (*cf.* Fig. 1, in Crozier and Pincus, 1926–27 (3, *a*)).

A litter of six rodless mice of an inbred "black—short ears—kinky tail—rodless" stock about 25 days of age was tested, together with an "albino—short ears—kinky tail—rodless" of similar age. A litter of two of an inbred "non-agouti—pink eyed—dilute—brown" stock of about 20 days served as controls.

Active adult animals with senses well developed do not orient on the inclined plane, naturally, but must be stimulated, and this stimulation must be given carefully and precisely. The mouse is placed

nose upward upon the incline and the tail gently pressed to the screen wire with a forefinger. The animal pulls hard to get loose. When the pressure is gradually increased the mouse pulls very hard. When the finger is suddenly released, the mouse runs forward quickly, taking the line of "least resistance" which determines the measured angel θ. A straight edge is placed between two marked points in the mouse's path and the angle measured with the transparent protractor.

III.

Six observations of θ were made in the dark for the rodless animals for $\alpha = 20°, 30°, 50°, 70°$ and for the controls at 15°, 30°, 50°, and 70°.

TABLE I.

Values of	15°	20°	30°	50°	70°
Black rodless *A*		51.2	62	70	74
" " *B*		50.4	61	71	77.8
" " *C*		52.8	61.4	72.4	79.4
" " *D*		51	59.2	71.4	78.6
" " *E*		52.4	62.8	73.4	80
" " *F*		50	62.2	74.4	80.6
Albino "		52.2	61.6	72.6	79.6
Control *A*	39.5		64	80	78.2
" *B*	44.4		55.5	68.4	80.4

TABLE II.

Values of	15°	20°	30°	50°	70°
Average θ, rodless		51.4	62.4	72.1	78.6
" θ, controls	41.9		59.7	76.2	79.3

The averages of the six observations for each animal are shown in Table I.

The averages for the rodless individual and for the controls are again averaged and these grand averages are given in Table II.

A 50 watt 115 volt white frosted Mazda light was then placed at the top of the plane and the animals were again tested at the previously recorded angles.

The averages of six observations for each rodless animal are given in Table III, together with their grand average.

When, however, the controls were tested in the presence of light, they utterly refused to mount the plane but turned quickly and moved away *from the source of illumination. Thus no records could be* obtained for them.

After about 5 minutes in the light to become adapted to the light, they were forced to walk up the plane facing the light and their tails were then pinched. I finally succeeded in getting the scattered

TABLE III.

Values of	20°	30°	50°	70°
Black rodless *A*	51.2	64	71.8	77.2
" " *B*	54.8	62	71.8	80.4
" " *C*	53.6	63	72.6	79.4
" " *D*	54.6	61.4	71.6	80.4
" " *E*	52.8	60.2	70.4	77.4
" " *F*	51.6	58.8	68	78.8
Albino "	49.2	60 8	71.6	79.8
Average	52.5	61.4	71.1	79.1

TABLE IV.

	20°	30°	50°	70°
Control *A*	42.8		63.2	58
" *B*	38.2	39.2	58.2	55.5

figures shown in Table IV. At 30° Control *A* refused entirely to mount the plane and the average for Control *B* at this angle is based upon only 4 observations.

IV.

It has been shown (Crozier and Pincus, 1926–27 (3, c)) that the behavior of young rats on an inclined plane in darkness is such that when θ is plotted against *log sin* α the curve tends to approach a straight line.

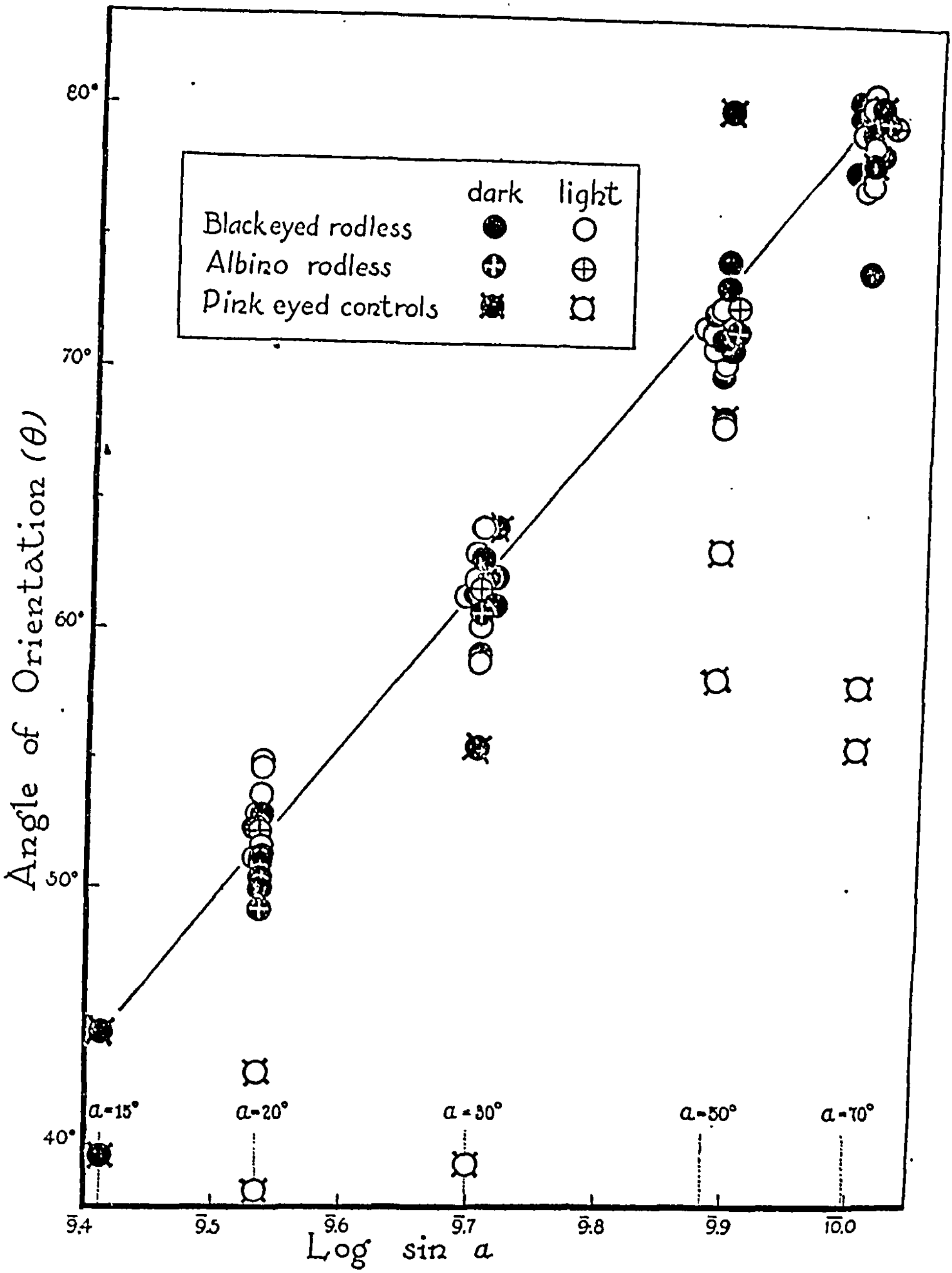

FIG. 1. The amounts of upward geotropic orientation of (θ) "rodless" mice are the same in darkness or in light. Mice with normal retina orient well in darkness, but in light their orientation is erratic. The plotted points are average measurements of the orientations of single individuals.

In Fig. 1 are plotted values of θ against the corresponding log sin α for the individual averages, as well as the scattered inaccurate values for the controls in the light (data of Tables I, II, III).

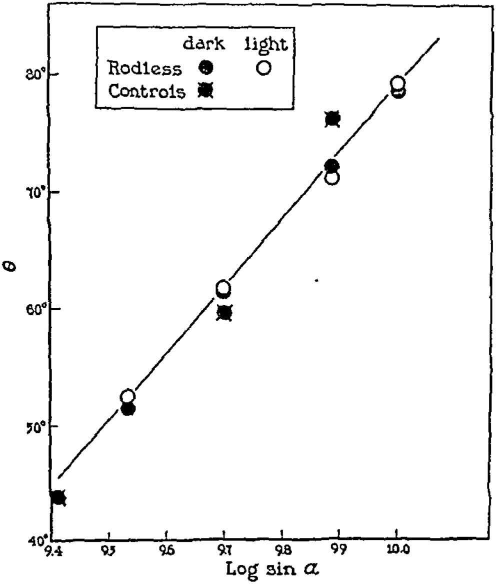

FIG. 2. Mean values for the amount of geotropic orientation (θ) of rodless and control mice in darkness or in light (rodless) is to a sufficient approximation a rectilinear function of the logarithm of the gravitational component in the plane of creeping (log sin α).

A line drawn through the general groups of points is such that when arbitrary values for points upon this line are substituted in the general relation,

$$\theta = K \log \sin \alpha - C$$
$$K = -62$$
$$C = 539.3$$

When a similar plot (Fig. 2) is made for the averages of (1) all rodless in the dark (Table II), (2) all rodless in the light (Table III), and (3) all controls in the dark (Table II), the result is even more striking. The geometric means are of a slant differing very little from that seen in Fig. 1.

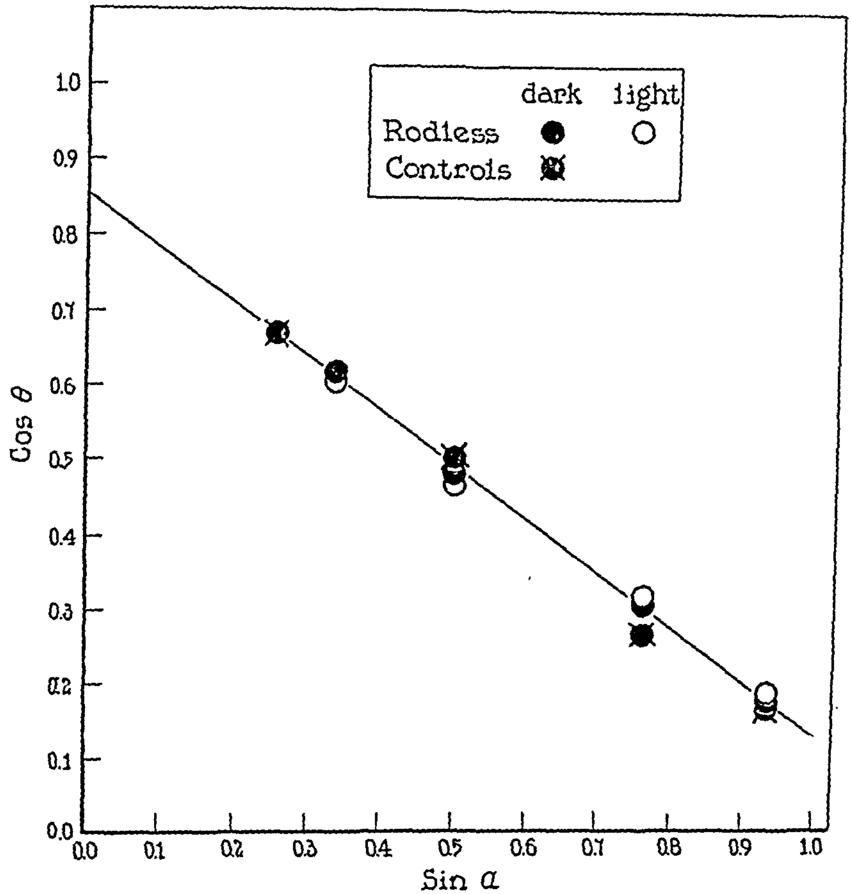

FIG. 3. A more exact relationship between the angle of upward orientation on the plane of creeping inclined at angle α to the horizontal is given by the rectilinear connection between cos θ and sin α.

Such geotropic orientation in the absence of other stimuli may be plotted with cos θ against sin α, in which case the curve should more nearly approach a straight line (Crozier and Pincus, 1926–27 (3, *c*)). Fig. 3 shows such a plot of the data recorded in Fig. 2.

DISCUSSION.

All three methods of handling our data show (1) greater variation of reaction for the controls in the dark, (2) very close agreement of reactions in the rodless animals in both light and darkness.

It has been shown (Crozier, 1925–28 (2)) that the photic orientation of certain animals, including young rats, is at an angle at which photic excitation upon the two sides are equal. It has been further demonstrated (Crozier and Pincus, 1926–27 (3, *a*)) that young rats with their eyes opened seek a darkened place in the field when light is introduced. The same workers (Crozier and Pincus, 1926–27 (3, *b*)) opposed phototropism to geotropism in the young rat, and found that the logarithm of the intensity necessary to counterbalance geotropic orientation bears a constant ratio to the logarithm of the angle of inclination $\frac{\log I}{\log \sin \alpha} = K$.

If the rodless animals are affected by the light, we should expect to see the values of θ observed in the light to be lower than those in the dark, which is not the case. Further experiments employing light of different intensities and wave-lengths are planned.

SUMMARY.

"Black—short ears—kinky tail—rodless" mice, controlled by "pink eyed—dilute—brown" mice, were tested on an inclined plane in order to determine if they are photically sensitive, and, if so, to get a quantitative expression for their visual receptivity. Rodless and control animals were tested in the dark to obtain an expression for normal geotropic orientation. Light was then introduced to modify these reactions if possible. Under light, the controls failed to orient, whereas the rodless gave reactions almost identical with those in the dark.

This test has failed in this experiment to suggest sight in the rodless mouse.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

XIII. The Temperature Characteristic for the Contraction Rate of the Whole Heart.

By ALFRED E. COHN.

With the Assistance of Yetta Porosowsky.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Accepted for publication, October 5, 1927.)

In a former paper in this series (1), Murray reported a study of the temperature characteristic of explants from auricles and ventricles of chicken embryos which was undertaken with a view to ascertaining whether a relation exists between the age of the embryo and this measurement. In the case of the explants he failed to find the relation. Fragments from hearts of embryos of the same age yielded different values and those of different ages, values which did not lend themselves to a characteristic arrangement. Murray suggested the possibility that if the observations were extended to a study of the whole embryo, in which the activities of all the structures were interrelated, greater uniformity of behavior might be detected. Crozier and Stier (2) have since reviewed Murray's conclusions. They doubt the probability of obtaining consistent values for the temperature characteristic μ in preparations such as Murray used in which the absence of a controlling focus permits the independent and alternating activity of a number of non-related pace makers, that correspond neither in their rates nor in their internal metabolic activities. They think significant results are less likely to be encountered in studying organs of intact animals, but "suggest their probable occurrence in the heart rhythms of embryos." They suspect that this is the state of affairs because in developing *Limulus* there is "different chemical control of heart pulsation in embryo and in adult *Limulus*," and because of "the relative diversity of pace-making control in the developing embryos."

Meanwhile experiments have been in progress designed to discover whether such changes exist in developing chicken embryos and to test the utilizability of the temperature characteristic in describing these changes in activity with age. This report has been delayed on account of difficulties encountered first in changing the temperature of the preparation uniformly and second in obtaining its measurement.

Technique.

The embryos were grown and their ages ascertained according to methods described in earlier papers in this series. To count the pulsation of the heart,

TABLE I.

The Temperature Characteristic of Embryo Chicken Hearts.

Age	No. experiments	Average rate of contractions*	Standard deviation ±	Average temperature characteristic	Standard deviation ±	Average temperature coefficient	Standard deviation ±
days							
3	7	182	19.87	12,400	1,100	2.1	0.2
4	11	191	27.10	12,700	1,970	2.3	0.2
5	6	210	52.60	12,900	1,590	2.1	0.2
6	9	221	11.70	10,800	1,640	1.8	0.15
8	2	220	12.49	8,700	270	1.5	0.1
10	8	239	9.64	8,300	1,500	1.6	0.1
11	2	213	5.47	7,400	1,000	1.5	0.7
12	4	224	10.29	8,600	1,400	1.6	0.1
13	1	245		7,600		1.5	
15	5	242	7.48	6,300	200	1.3	0.04
18	1	200		7,300		1.5	

* At 38°C.

small windows were cut in the shell, care being taken to avoid hemorrhage; if visible hemorrhage occurred the embryo was discarded. The egg was placed on the stage of a microscope and the whole enclosed in a small thermostat, the temperature of which was altered by the opening and shutting of one or more of several doors. Petri dishes full of water were kept in the chamber. The eggs lost no weight in the operation. The site at which pulsations either of the heart itself or of a prominent vessel, were counted was illuminated by a beam of light brought to a focus at that point. The light passed through a chamber in which water was kept flowing, to absorb heat.

The temperature to which the embryo and the heart itself were exposed was

measured with a thermocouple and galvanometer devised by Clark (3). By the use of this device the accuracy is correct to 0.04°C.; the temperature could be taken at a distance only 1 to 2 mm. removed from the heart. The lag often encountered in experiments in which the sensitiveness of the heart to change in

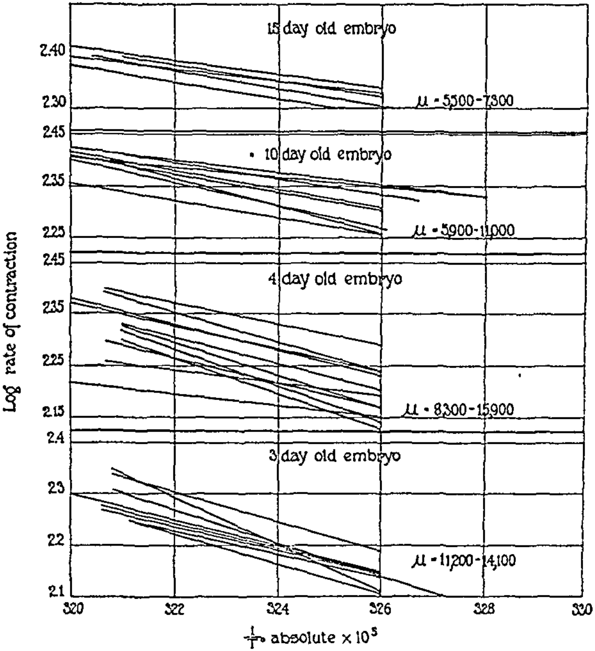

FIG. 1. The relation of the contraction rate to the temperature is presented for all the experiments performed in embryos 3, 4, 10 and 15 days old.

temperature can be detected by a change in its rate before the mercury thermometer registers the new level, is avoided. Counts of the heart rate were made several times both with rising and falling temperature for half minutes, the intervals being sounded by an electric buzzer in series with a chronograph. Cases in which there was conspicuous lack of uniformity of behavior were omitted

from the series. On account of the rapidity of the temperature adjustment the duration of the observation could be correspondingly brief. To avoid injury the temperature range was confined to limits between 33° and 39°C. This range is short, but to exceed it might subject the hearts to injury and as a result interfere with repeating the observations.

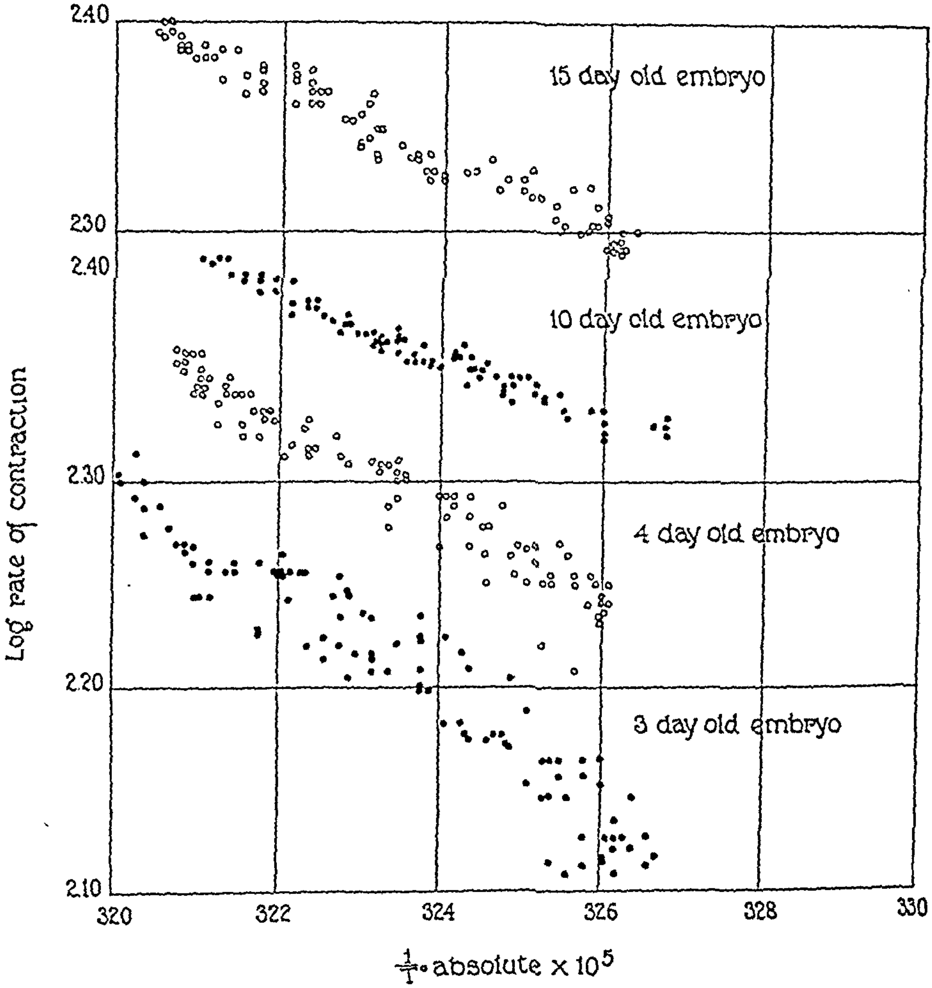

FIG. 2. The relation of the contraction rate to the temperature is shown for one experiment each in embryos 3, 4, 10 and 15 days old.

For the calculation of the temperature characteristic the equation of Arrhenius was used in the form given by Murray (1):

$$\mu = 4.61 \frac{\log K_2 - \log K_1}{\frac{1}{T_1} - \frac{1}{T_2}}$$

Observations.

Observations were made in embryos 3, 4, 5, 6, 8, 10, 11, 12, 13, 15 and 18 days old (Table I). The results obtained at ages of 3, 4, 10 and 15 days are reproduced graphically in Fig. 1. Except at the age of 4 the curves at each age present a fair degree of similarity. The details of a single experiment at each of these ages is given in Fig. 2. As in the case of Murray's experiments there was at a given age no striking uniformity; in two cases the fluctuations were as wide (8,300 to 15,900 μ) as that which he found in fragments of 8 day auricles. In general there appears to be a reduction in the temperature characteristic, whether the highest, lowest or the average values for each age are compared (Fig. 1). Instances in which μ = 14,000 were found in the case of 3 and 4 day embryos only, but in four only of the eighteen experiments.

The rate of the heart at 38°C. was taken in each case; the averages are given in Table I. There appears to be a slight change with time. The results exhibit a considerable difference between these and those found in a former series (4). The difference may be due to three factors, at least. In the earlier experiments, temperature regulation was in the first place less satisfactory than in the present series, the thermometer was read at a distance farther removed from the heart and there was a certain amount of evaporation of water from the egg in the course of the observation. These errors were all avoided in the present series. When the curves for heart rate seen in these experiments were extended upward to 40°C. and downward to 30°C., theoretical contraction rates were obtained so that the temperature coefficient Q_{10} might be calculated. In this there was a slight fall though somewhat irregular from 2.1 at 3 days to 1.5 at 18 days. Obviously the hearts exhibited much more uniform behavior than did the auricular fragments (1).

DISCUSSION.

The curves which serve as a basis for calculating the temperature characteristics in intact chicken embryos approach more closely to the form usually found in such calculations than do those taken from fragments of the auricles and ventricles. Curves drawn through the

marginal rates (2) are parallel with the best average curve. Although there is less variation at a given age in the undisturbed hearts than in fragments taken from them, a certain amount of variation nevertheless exists. Whether this variation is due, as Crozier and Stier (2) suggest, to "diverse pace-making processes in the hearts of different embryos," cannot of course yet be known. Their mention of the possibility occurs in connection with isolated heart preparations. These experiments indicate that at a given age, even when the rates at 38°C. are similar, there may be variations in the temperature characteristic. That the

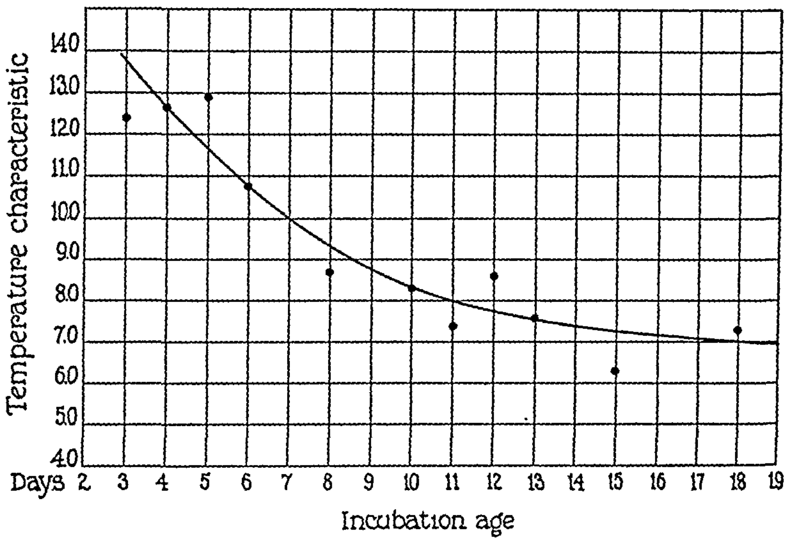

FIG. 3. A curve indicating the alteration of the temperature characteristic, μ, with time is shown. It represents averages of all the data at each age. It exhibits a systematic change although at a given age the range of individual observations is large.

fluctuations are not great in most instances is apparent. A striking point consists in the manner in which a change with age takes place (Fig. 3). There is perhaps too great a range of values at any age to permit the data to do more than suggest this possibility. But that this result might be anticipated arises from considering the changes in rate which occur and from a knowledge, though still incomplete and inexact, of the organization of heart muscle tissue which is synchronously taking place. The results obtained are otherwise not inconsistent with those collected by Crozier (5).

SUMMARY.

The relation of heart rate frequencies to temperature in intact chicken embryos has been studied and the temperature characteristics calculated for each of a number of ages. These have been found to vary from 14,000 or better 12,000 μ for embryos 3 days old, to about 6,000 μ for others of 15 days. There appears to be a systematic change with time. If this inference is correct, important correlation with other properties of the pace-making function in the intact heart should become possible.

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THE DETERMINATION OF THE EQUIVALENT WEIGHT OF PROTEINS.*

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According to Hoffman and Gortner (1) proteins combine stoichiometrically with acids and bases only within a limited pH range, which he places approximately between the limits 2.5 and 10.5. At pH values below 2.5 acid is still increasingly bound, but the binding in this region follows adsorption laws. Bases, above a pH of 10.5, behave in an analogous manner. This conclusion is based on the facts that (*a*) at no pH does the amount of acid or alkali bound seem to become constant and independent of further pH change, and (*b*) the temperature coefficient of acid and alkali bound begins to manifest itself to a significant degree at pH values below 2.5 and above 10.5 respectively.

Naturally it is difficult to obtain accuracy in electrometric titration at low or high pH values, where a small change in the ratio of hydrogen ion concentration means a large actual change. Regarding the conclusions drawn from temperature coefficient, no account is taken of the possible temperature effect on the difference in the activity of hydrogen ion in pure water and in protein solution, an effect which should at least be considered before too definite conclusions are drawn.

Jordan-Lloyd and Mayes (2) are in general agreement with the above conclusion from work done on the titration of gelatin with HCl. They state that up to a concentration of 0.04 normal acid a typical titration curve is obtained, but that above this concentration more acid begins to be bound.

Other workers find, at least in the case of the binding of HCl by

* This work was carried out in the chemical laboratory of Stanford University, and the author wishes to acknowledge his appreciation of the courtesy of the department extended to him as visitor.

gelatin, a typical titration curve showing no discontinuous section even in solutions considerably more acid than 0.04 normal (3, 4).

Volumetric conductivity titrations have been suggested from time to time as a means of obtaining the equivalent weight of proteins (5, 6, 7), but in only one case does the method seem to have been directly applied. Hitchcock (8) titrated gelatin with various acids and obtained concordant values for the equivalent weight of gelatin which agreed substantially with values obtained by electrometric titration.

The question arises as to whether any light can be thrown on the generality of Gortner's claim of a type of binding, easily observable at high or low pH values, different from that which one finds through the intermediate pH range. Doubtless a certain amount of "peptid-linkage" binding will take place, but does it occur to the extent assumed by Gortner?

Theoretical Considerations.

If we are interested in the increase in conductance of a titration mixture to which quantities of acid are being added after the stoichiometrical end-point has been reached we have the following rough relationships in the case of protein titrations.

Starting from a point where hydrolysis has been largely repressed (n, Fig. 1), we have for the change in λ, the conductance corrected for volume change during titration, with x, the number of equivalents of acid added,

$$\frac{d\lambda}{dx} = k\,\Lambda_0 \tag{1}$$

in which the constant k depends on cell constant and degree of ionization, and Λ_0 is the equivalent conductance of the acid in ionic form. This relation holds only in case no appreciable "adsorption" takes place. In the latter case we have

$$\lambda = \lambda_0 + k\,\Lambda_0\,(x - n) + k'\,p\,\Lambda_0\,nu \tag{2}$$

where k has the same significance as above, k' has analogous signifi-

cance for the protein salt, n is the number of equivalents of acid bound by adsorption, u is the transference number of the protein salt anion, and λ_0, the *integration constant of equation* (1) (*and also of equation* (5)), is the conductance of the mixture at the stoichiometrical end-point, corrected for hydrolysis. p is defined by the ratio $\frac{\Lambda_0 \text{ of protein salt}}{\Lambda_0 \text{ of acid}}$ so that

$$\Lambda_{0\,\text{protein salt}} = p\,\Lambda_{0\,\text{acid}}$$

But, though n is not, strictly speaking, a function of x, if we confine ourselves to conditions such that the increase in hydrogen ion concentration is nearly proportional to the amount of acid added, then, if n_0 is a constant and b the adsorption exponent,

$$n = n_0\, x^b \tag{3}$$

Substituting (3) in (2) we have

$$\lambda = \lambda_0 + k\,\Lambda_0\,x - n_0\,\Lambda_0\,(k - k'\,pu)\,x^b \tag{4}$$

Both u and p are fractions, the latter being *rather small in general*, and therefore, since k will not differ greatly from k',

$$k > k'\,p\,u$$

so that

$$\lambda = \lambda_0 + k\,\Lambda_0\,x - \text{const.} \times x^b$$

and

$$\frac{d\lambda}{dx} = k\,\Lambda_0 - b\,\text{const.} \times x^{b-1} \tag{5}$$

The two slopes differ therefore by the quantity $-$ const. x^{b-1}, where the constant is in all cases positive. In practically all cases reported by Hoffman and Gortner b is less than 1, or $(b-1)$ is negative. Thus for very high values of x, assuming no disturbing complications, the two slopes become identical.

If we are titrating a given amount of acid with a protein solution,

and if we study the same portion of the titration curve as above, *i.e.* with acid still in goodly excess, we have, if no adsorption takes place,

$$\lambda = \lambda'_0 - k\ (\Lambda_{0_{\text{acid}}} - \Lambda_{0_{\text{protein salt}}})\ x'$$

where λ'_0 is the conductance of the acid solution before any protein is added.

Putting in

$$\Lambda_{0_{\text{protein salt}}} = p\ \Lambda_{0_{\text{acid}}}$$

we have

$$\lambda = \lambda'_0 - k\ \Lambda_0\ (1 - p)\ x' \tag{6}$$

or

$$\frac{d\lambda}{dx'} = -\ k\ \Lambda_0\ (1 - p) \tag{7}$$

where x' is the number of equivalents of protein added in titration.

In case of appreciable adsorption we have

$$\lambda = \lambda'_0 - k\ \Lambda_0\ (1 - p)\ (x' + nu) \tag{8}$$

Under such conditions, if c be the number of equivalents of acid originally present, we cannot represent the concentration of free acid by the quantity $(c-x')$ as we could if there were no adsorption. The free acid will be $(c-x'^a)$ where the exponent a takes account of adsorbed as well as neutralized acid.

Thus,

$$n = n_0\ (c - x'^a)^b \tag{9}$$

and

$$\lambda = \lambda'_0 - k\ \Lambda_0\ (1 - p)\ x' - k\ \Lambda_0\ (1 - p)\ un_0\ (c - x'^a)^b \tag{10}$$

so that

$$\frac{d\lambda}{dx'} = -\ k\ \Lambda_0\ (1 - p) + bk\ \Lambda_0\ (1 - p)\ un_0\ (c - x'^a)^{b-1}\ .\ ax'^{a-1} \tag{11}$$

In this case the two slopes differ by the quantity const. x'^{a-1} $(c-x'^a)^{b-1}$, where the constant is in all cases positive. As x' increases

$(c-x'^a)$ diminishes and, since $a>1$ and $b<1$, these two curves will tend to diverge in place of becoming parallel, as was the case with increase of x in titrating protein with acid.

These tendencies are shown by the diagrammatic graphs in Fig. 1. Curve A represents titration of a sample of protein with acid and Curve B the titration of a sample of acid with protein. Let the dotted lines represent the experimental curves. If we assume that appre-

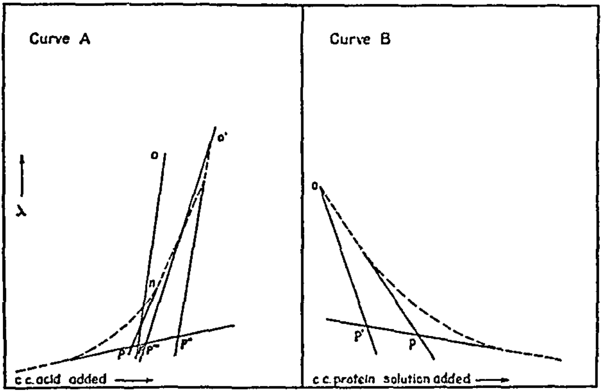

FIG. 1. Curve A represents the change in conductance when protein is titrated with acid, while Curve B gives the corresponding change when acid is titrated with protein. Analogous curves might be drawn to represent behavior toward bases. The dotted line represents a hypothetical experimental curve. For explanation of the various end-points, P, P', P'' and P''', see body of text.

ciable adsorption has taken place we may, on the basis of the above equations, draw in hypothetical arms, OP' in both curves, representing the curve one would get if no adsorption took place, *i.e.* if chemical neutralization were the only type of binding. Thus the point P is the observed end-point, P' is the hypothetical end-point which would give the stoichiometrical equivalent weight of the protein, and in case the titration were carried to the point where the two arms become parallel, the observed end-point would shift to P''.

It will be seen that if one calculates the number of cc. of standard alkali, or in this case acid, which is equivalent to 1 gm. of protein, one will not, in general, get the same result when titrating protein with acid as when titrating acid with protein unless adsorption is negligible. When titrating protein with acid (Curve A), points P and P' are rather close together since they are bound to occur at or near the

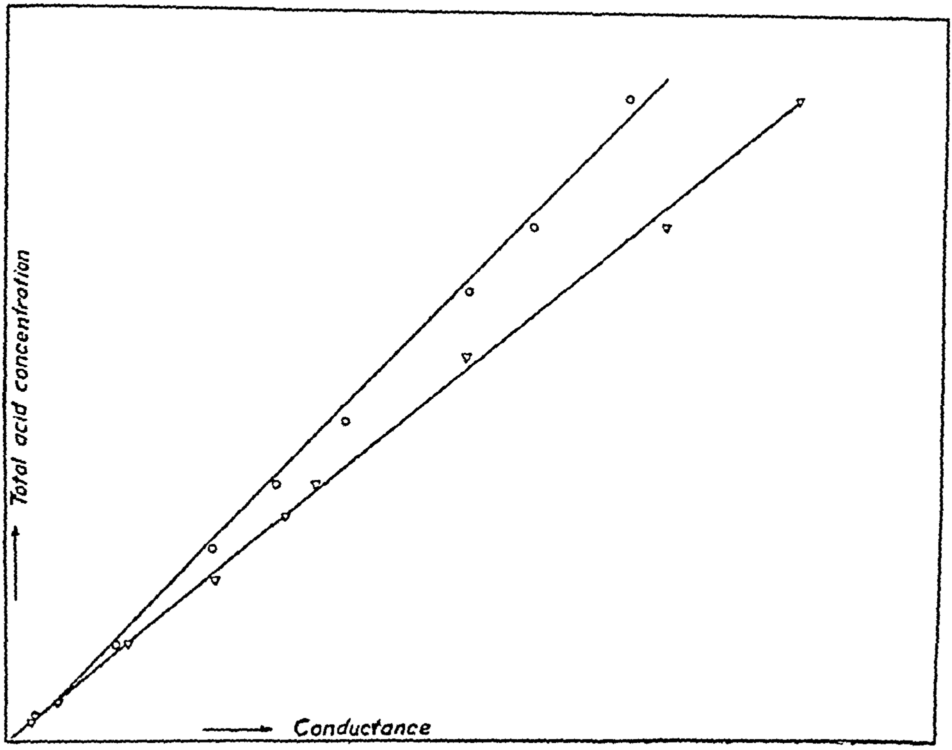

FIG. 2. Showing the difference in slope of conductance titration curves *after the stoichiometrical end-point has been passed* (I) when no adsorption takes place, and (II) when appreciable adsorption takes place. The curves are plotted from data calculated from results by Hoffman and Gortner (1) on the addition of HCl to water and to 1 per cent teozein solutions at 15°C. respectively.

point of convergence of the real and hypothetical arms. Thus, unless one goes far enough in one's titration to reach P'' as end-point, which is quite unlikely,[1] the observed end-point and the point which would give

[1] If one did get into this region one would probably imagine he should be getting a straight line and either disregard the particular experiment as untrustworthy or else arbitrarily take some average slope OP'''.

the stoichiometrical equivalent weight of the portein will lie rather close together.

Such is not the case (Curve B) when titrating acid with protein. In such a case the points P and P' will be read far from the point of convergence of the two arms. The estimated equivalent weight from the observed end-point P should therefore differ appreciably from that estimated from P' if appreciable adsorption takes place. Thus the observed value obtained by titrating protein with acid should be lower than that obtained by titrating acid with protein.

The same reasoning and predictions would apply also to the binding of bases.

The question whether the difference due to adsorption, on the basis of the results of Hoffman and Gortner, is large enough to warrant the above consideration may be easily answered affirmatively since they indicate, if their conclusions are valid, that over 90 per cent of the acid-binding and over 95 per cent of the alkali-binding is due, in the case of their prolamines, to adsorption. From their electrometric titration data on teozein at 15 degrees the conductance curves in Fig. 2 are plotted. These show the actual difference in slope between the curve obtained when HCl is added to water and that when it is added to completely "neutralized" teozein in 1 per cent solution.[2] The electrometric titrations were carried down nearly to a pH of 0.5.

The present paper presents data indicating that, in the case of gelatin and HCl, the same value is obtained for the equivalent weight of gelatin whether the gelatin is titrated with acid or the acid with gelatin. In case of gelatin and NaOH, contrary to obtaining a higher equivalent weight for the gelatin when titrating the base with the protein, a slightly lower value was obtained, due probably to carbon dioxide absorption.

[2] Plotted conductances were obtained by multiplying the various ion concentrations by their respective ion conductances at 15 degrees and adding. The value 25 was assumed for the protein ion conductance. The hydrogen ion concentration was obtained from pH values, the chloride ion concentration from the sum of hydrogen and total bound acid, the latter being calculated from data on page 336 (1), and the protein ion concentration was assumed equal to the second named component of the chloride ion concentration. The significant fact is that there is a real difference in slope through a pH range below that at which all of the protein has been "neutralized."

EXPERIMENTAL.

Since it is impracticable to titrate acid or base with gelatin without considerable volume change, all four titrations reported were made with about the same volume change, and the various conductances were corrected to the original volumes of the corresponding solutions. A preliminary pair of titrations first with a fairly concentrated, though unstandardized, acid and then with the same acid diluted to one-tenth of its original concentration gave the same end-point, when volume corrections were made, in equivalents of acid bound per gm. of gelatin.

TABLE I.

Acid added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 75 cc.	Gelatin added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 77 cc.
cc.			*cc.*		
0	105.0	105.0	0	3366.0	3366.0
1	154.0	156.0	1	3001.0	3040.0
3	213.1	221.6	2	2632.0	2700.0
5	282.7	301.6	3	2302.0	2392.0
7	388.0	424.0	4	2003.0	2107.0
9	562.0	629.7	5	1762.0	1877.0
11	785.0	900.0	6	1569.0	1691.0
13	1027.0	1205.0	7	1427.0	1557.0
15	1266.0	1519.0	8	1336.0	1475.0
17	1501.5	1842.0	10	1249.0	1411.0
20	1835.5	2325.0	12	1233.0	1425.0
			15	1256.0	1501.0
			17	1286.0	1570.0
			19	1311.5	1635.0
			20	1323.0	1667.0

The first three columns give the data obtained when different amounts of 0.1175 normal HCl were added to 1 gm. of gelatin in an original volume of 75 cc. The last three columns give corresponding data obtained when a solution of gelatin containing 0.0385 gm. per cc. was added in varying amounts to a solution containing 2 cc. of 0.1175 normal HCl in an original volume of 77 cc.

The titrations were made in a constant temperature bath thermally regulated. No adjustment to a particular temperature was made but the value 25.65° ± 0.05°C. was maintained.

A 1 per cent solution of the gelatin gave to water of specific conductance 3×10^{-6} (at room temperature) a pH of 4.90 and a specific conductance of about 100×10^{-6}. A sufficient quantity of gelatin solution was made up for all four titrations so that the magnitude of the correction necessary to bring it to its isoelectric point would be the same in all cases and thus the results of titrating

gelatin with acid and acid with gelatin could be compared regardless of the uncertainty of any correction. The magnitude of the correction was read from an independent electrometric titration curve (4). (See also (8)).

Data are given in Tables I and II and the results in Table III. The data for

TABLE II.

Base added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 77 cc.	Gelatin added	$k \times 10^6$ measured	$k \times 10^6$ corrected to 77 cc.
cc.			*cc.*		
0	105.0	105.0	0	2140.0	2140.0
1	127.3	129.0	1	1989.0	2015.0
3	163.5	170.0	2	1847.0	1895.0
5	241.7	257.4	4	1580.0	1662.0
7	368.0	401.5	6	1364.0	1470.0
9	523.0	584.0	8	1208.5	1334.0
11	683.0	780.5	10	1109.0	1253.0
13	839.0	980.4	12	1064.0	1229.5
15	989.0	1182.0	14	1054.0	1246.0
17	1134.0	1384.0	16	1068.0	1290.0
20	1341.0	1689.0	18	1098.0	1355.0
			20	1140.0	1436.0
			23	1199.0	1557.0
			26	1260.0	1685.0
			30	1324.0	1840.0

The first three columns give the data obtained when different amounts of 0.1219 normal NaOH were added to 1 gm. of gelatin in an original volume of 77 cc. The last three columns give corresponding data obtained when a solution of gelatin containing 0.0385 gm. per cc. was added in varying amounts to a solution containing 2 cc. of 0.1219 normal NaOH in an original volume of 77 cc.

TABLE III.

Experiment	End-point (cc. titrating sol. obtained from curves)	0.1 N acid or base per gm. gelatin (uncorrected)	pH original gelatin sol.	Correction 0.1 N acid or base to isoelectric point	Corrected value of cc. 0.1 N acid or base per gm. gelatin
		cc.		*cc.*	
Titration of gelatin with acid	8.15	9.58	4.90	−0.65	8.93
" " acid with gelatin...	6.40	9.54	4.90	−0.65	8.89
" " gelatin with base	5.50	6.70	4.90	0.65	7.35
" " base with gelatin	9.15	6.93	4.90	0.65	7.58

the acid titrations are plotted in the graphs of Fig. 3, and those for the alkali titrations are plotted in those of Fig. 4.

A solution of 7.70 gm. dry gelatin in 200 cc. was prepared by dissolving the gelatin in warm water, cooling and making up to volume. At such a concentra-

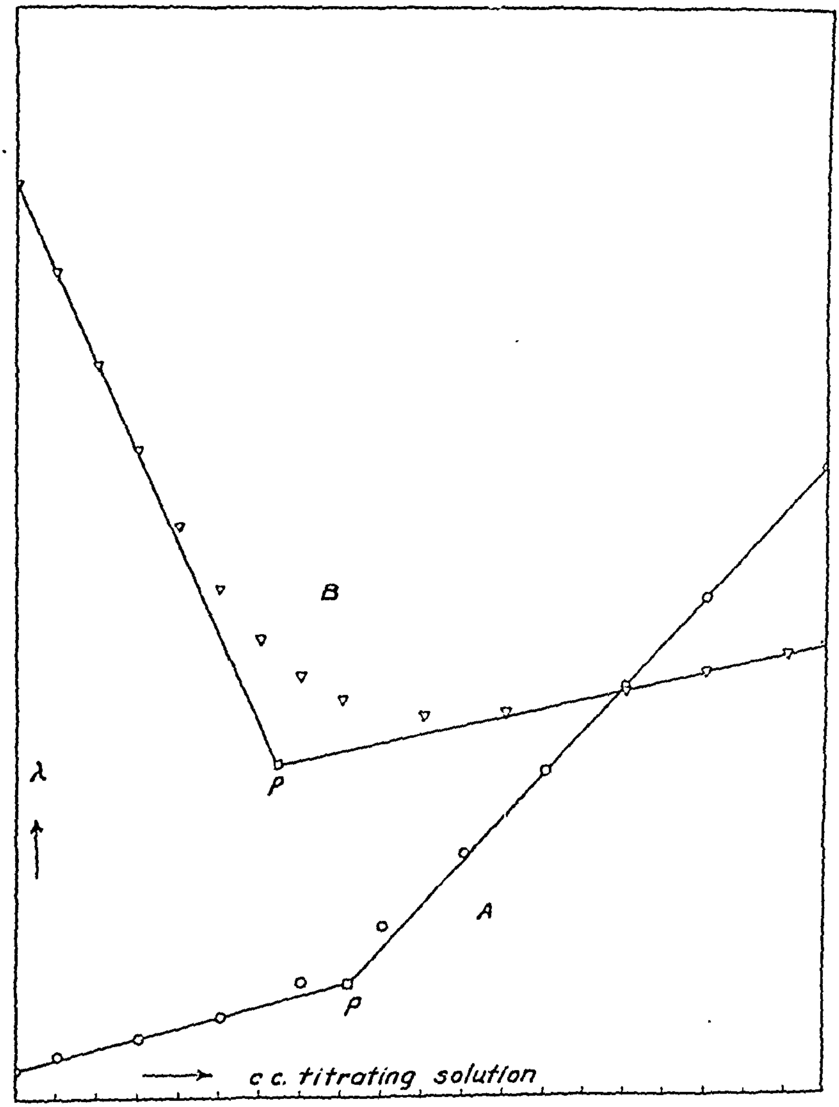

Fig. 3. Conductance titration curves of gelatin and HCl. Curve *A* is for titration of gelatin with acid and Curve *B* is for the titration of acid with gelatin solution. Conductances, corrected to original volumes are in both cases ordinates, while cc. of acid or of gelatin solution added are respective abscissæ.

tion the solution, upon standing for considerable time, would set to a gel at room temperature, but when freshly prepared it could be added from a burette for some time, even at room temperature. In making the titrations with the gelatin solutions, care was taken to obtain proper draining of the rather viscous liquid so as not to introduce appreciable volume error into the titration. For titration of the gelatin with acid or base, 26 cc. of this gelatin solution, which contained almost exactly 1 gm. of the dry gelatin, were used as samples. The standard acid was an HCl solution of normality 0.1175, and the base, prepared by diluting a 50 per cent NaOH solution from which the carbonate had settled with CO_2-free water, had a normality of 0.1219.

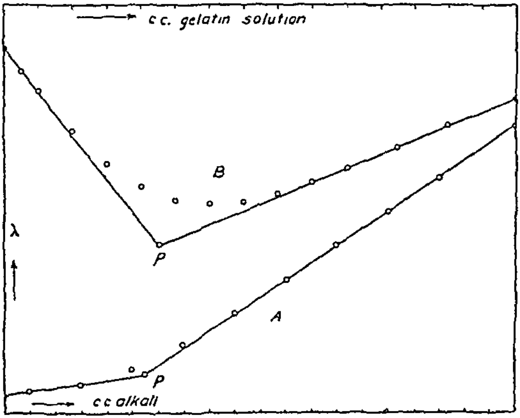

Fig. 4. Conductance titration curves of gelatin and NaOH. Curve A is for titration of gelatin with base, and Curve B is for the titration of base with gelatin solution. Conductances, corrected to original volumes are in both cases ordinates, while cc. of base or of gelatin solution added are respective abscissæ. (In this figure the abscissæ scale for Curve B is two-thirds that for Curve A.)

DISCUSSION AND SUMMARY.

The magnitude of the correction in the fifth column of Table III may be open to some doubt, as are all corrections of such a character, and the significance of the above experiment in the author's mind lies

not so much in the actual magnitude of the values given in the last column of this table as in their comparative magnitudes. For this reason the entire experiment reported was performed in a single session[3] using the same gelatin solution, so that, whatever the magnitude of the correction, it would be the same in all cases.

Actually the results in the case of the acid titrations are in fair agreement with those of Hitchcock (8). In the present experiment it is seen that, within the limits of experimental error, one gets the same value for the number of cc. of tenth normal acid bound by 1 gm. of gelatin whether one titrates with the acid or with the gelatin. In the case of the base there is a small difference, due probably to carbon dioxide, but this effect is in a direction opposite to that which one would expect on the assumption that it is due to appreciable adsorption.

From this it is concluded that the binding due to adsorption in the case of gelatin is not significant compared to that due to chemical neutralization. The author realizes that gelatin is a poor choice for a basis of generalizations, and similar work is at present in progress on various other proteins. He does feel, however, that the conclusions of Hoffman and Gortner from their work on the prolamines may also be too widely generalized, and that, on the whole, the acid or alkali bound by adsorption in the case of proteins will not constitute the large majority of the total amounts bound, though certainly one will expect a certain amount of such binding in all cases. It also seems that before placing undue emphasis on the conclusions of these workers the possibilities of equivocal results due to specific technique should be considered. This technique consisted in introducing weighed amounts of dry protein into a definite volume of standard acid or base at the equilibrium temperature, in general, and, "after about 15 minutes, during which time the flask was shaken several times," determining the pH of the equilibrium solution. Is it possible that the actual speed of solution of the protein is such that, even though reproducible results are obtained using identical technique, actual equi-

[3] The experiment reported is one of four performed. It may be stated that the last three gave substantially the same results, the first experiment being the only one yielding peculiar results. These peculiarities were found to be due in the main to insufficient care in titrating with the viscous gelatin solution.

librium conditions are approached only when comparatively high concentrations of acid or alkali are employed, in which cases the solution velocity of the protein may be expected to be greater, other factors remaining constant?

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PROTOPLASMIC ASYMMETRY IN NITELLA AS SHOWN BY BIOELECTRIC MEASUREMENTS.*

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(Accepted for publication, October 16, 1927.)

The study[1] of bioelectric phenomena has been hampered because nothing could be measured except the potential difference between selected spots and it has been impossible to determine the value of the potential difference across the protoplasm at any one spot.[2] Since it is highly desirable to obtain such values, an attempt was made to do so.

In the case of *Valonia*[3] this was done by piercing the cell with a glass capillary filled with sap and leading off from the interior of the capillary to a point on the exterior of the cell, so that the circuit passed only once through the protoplasm and the electromotive force was wholly due to the protoplasm at the spot where the external contact was made (Fig. 1).

In the case of *Nitella* such a procedure is much more difficult owing to the smaller diameter and greater delicacy of the cells.[4] Although experiments have been made by this method at various times during

* The authors desire to express their gratitude to the Carnegie Institution of Washington, D. C., which generously provided for the beginning of these investigations (1922–25).

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83.

[2] For example, in leading off with identical solutions from two points we often obtain a reading of zero when in reality each point may have a potential difference of 30 millivolts or more.

[3] *Cf.* Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, xi, 193.

[4] Regarding the technique of this method see Nichols, S. P., *Bull. Torrey Bot. Club*, 1925, lii, 351. The method has been employed by Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., *Carnegie Inst. Washington Year Book*, 1925–26, xxv, 248).

the last 5 years the writers prefer the method about to be described on account of its rapidity and certainty. With this method we arrive at the same result by reducing the potential difference across the protoplasm at one spot approximately to zero by killing it in such fashion that during the time of the experiment the injury does not

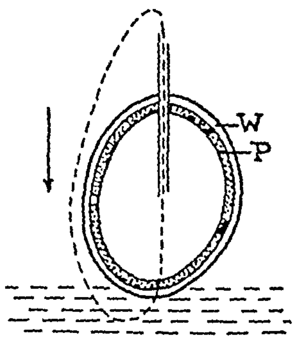

FIG. 1. Diagram of a cell of *Valonia* which is pierced by a glass capillary so that the protoplasm adheres to the glass and forms an electrical seal which prevents a short circuit through the cell wall.[3] The arrow shows the direction in which the positive current tends to flow when cell sap is applied to the exterior of the cell. *W*, cell wall; *P*, protoplasm.

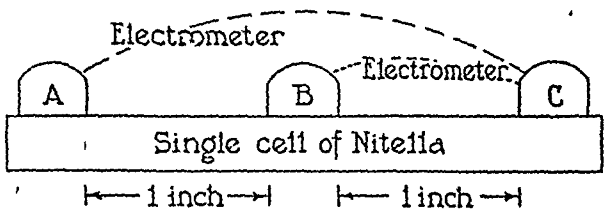

FIG. 2. Diagram of an experiment in which *A* and *B* are connected to *C*.

appear to affect other points at a sufficient distance in the same cell. When we lead off from the killed spot to a normal region, the circuit passes once through dead and once through living protoplasm and the results justify the conclusion that under proper conditions the observed value is practically all due to the potential difference across the living protoplasm at the uninjured spot[5] (Figs. 2 and 9).

[5] This method might not succeed so well with *Valonia* on account of the short-circuiting in the cell wall.[3]

The experiments were made upon carefully selected material of *Nitella flexilis*.[6] Cells up to 4 or 5 inches long were taken from the central portion of the plant. The larger adhering cells were trimmed away and the cells allowed to stand for

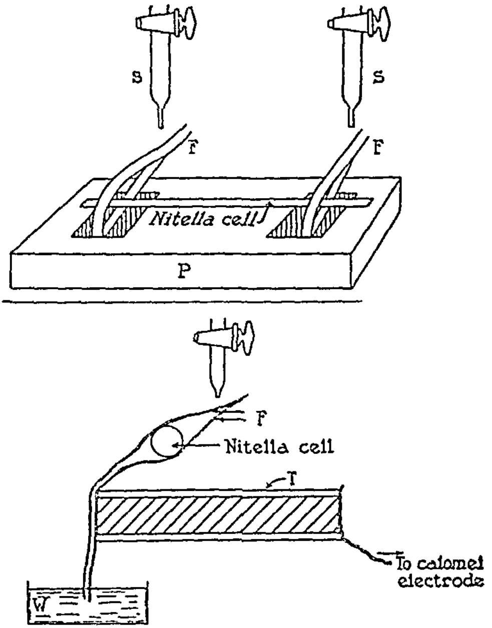

FIG. 3. Diagram to illustrate the manner of leading off from the cell, which rests on a paraffin block *P* having two or more openings (two only are shown here) above each of which is a stop-cock *S* from which a solution runs down upon a double strip of filter paper *F* which passes on both sides of the cell and descends through the opening to a waste jar *W*, just above which it encounters a tube *T* filled with agar (made up with saturated KCl) which is connected with a string moistened with saturated KCl) running to a calomel electrode.

[6] Kindly identified by Dr. J. S. Karling.

an hour or more in tap water before being used. Only normal cells in good condition were employed and every care was taken not to subject them to unfavorable influences during the course of the experiment (except for the application of the toxic agent at the spot selected for killing).

In making an experiment a single multinucleate cell was placed upon a paraffin block *P*, as shown in Fig. 3. The block had two openings at each of which a strip of filter paper *F* was in contact with the cell. Each strip was divided into two parts, one passing over and the other under the cell, so as to make contact on both sides. Above the cell the filter paper was connected with a reservoir so arranged that on opening a stop-cock *S* a constant stream of solution flowed over the filter paper[7] (on both sides of the cell) and down through the opening to a waste jar *W*.[8] Just below the cell the filter paper came in contact with a tube *T*[9] filled with agar made up in saturated KCl, which ran to a vessel filled with saturated KCl into which dipped a calomel electrode filled with 2 M KCl. This was the procedure with solutions in general but in experiments with sap contact was made by a piece of absorbent cotton soaked in sap which could be replaced by another soaked in sap containing chloroform.

The contacts at *A* and *C* were not near enough to the ends of the cells to involve certain minute cells which are present at the very end but even if these cells had been involved it does not seem probable that they could interfere with the results under the conditions of the experiment.

In preparing for an experiment the cells were freed from adhering drops of water by drying them lightly with filter paper before placing them on the paraffin blocks. Sufficient space was left between the contacts on the cell to prevent undue short-circuiting: this space could be an inch or more in length in cells 4 or 5 inches long. In order to prevent the cells from drying out, cotton wet with distilled water was placed at certain points;[10] in no case was the cell allowed to suffer from lack of water and it was carefully watched for signs of injury throughout the experiment.

The experiments were made at room temperature varying from 20 to 25°C., but the variation in any one experiment was less than 1°C.

The apparatus used to measure the potential differences had to fulfill the requirements of being an electrostatic instrument with a very short period. Such an instrument was obtained by combining a thermionic vacuum tube with a string

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[7] In the case of living protoplasm the variations in the rate of flow occurring in these experiments did not affect the results but in the case of dead or injured protoplasm (where sap comes out) the rate of flow is of importance. There seems to be no noticeable effect due to "Strömungselektricität," with a gentle flow.

[8] Reservoirs and waste jars must be carefully insulated to prevent short-circuiting.

[9] This was placed below in order to prevent the KCl from contaminating the solutions in contact with the cell. The agar was employed to prevent the solutions from being washed out of the tube.

[10] This did not noticeably increase the tendency to short-circuiting in the cell wall (regarding this, see Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83).

galvanometer. A similar arrangement has been used by Forbes[11] in the measurement of action currents of nerves, but the apparatus employed here is more like the second stage of that described by Chaffee.[12] In our work the vacuum tube was not necessary for the purpose of amplification, but only for converting the string galvanometer into an electrostatic instrument. The potential was applied to the grid and filament circuit of the vacuum tube, and the changes were recorded by the string galvanometer, the image of the string being projected and photographed on bromide paper.

A diagram of the circuit used is shown in Fig. 4. *V* is a vacuum tube; the type used in this work being an RCA 201A. *B* is a battery of 80 or 90 volts, about one-half of it provided with 1.5-volt taps in order to obtain small steps in voltage for balancing purposes. With the filament lighted, and the switch *S* thrown to the right (connecting grid to filament through the *C* battery), the clip *T* is at-

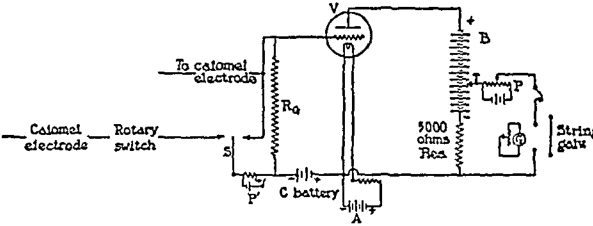

FIG. 4. Diagram of the electrometer: *V* is a vacuum tube (with batteries *A*, *B*, and *C*). The circuit can be closed through the wall galvanometer *G* or through the string galvanometer. A grid leak R_G is sometimes used: the potentiometer *P′* is used for calibration.

tached to one of these taps so that the galvanometer *G* reads zero or nearly so. The final adjustment to zero is made by means of the potentiometer *P*. This potentiometer has about 700 ohms resistance, and is connected across two dry cells in series, thus furnishing a total of 3 volts in small steps. This acts as a vernier adjustment on the tap *T*. The galvanometer *G* is a moving coil Leeds and Northrup wall type, and is switched into the circuit and balanced to zero before switching to the string. A double pole, double throw switch is provided for switching from the wall galvanometer to the string galvanometer and *vice versa*.

For the measurements in this work it was necessary to decrease the sensitivity

[11] Forbes, A., and Thacher, C., *Am. J. Physiol.*, 1920, lii, 409.

[12] Chaffee, E. L., *J. Optical Soc. Am.*, 1923, vii, 1.

of both galvanometers. In the case of the wall galvanometer this was done by shunting it with a variable resistance and in the case of the string galvanometer the string was tightened and some resistance was put in series with the string. The tension of the string was such that when a potential of 1 millivolt was applied to it directly a deflection of 1 or 2 mm. was obtained on the record. The string galvanometer was Type *A* as made by the Cambridge Instrument Co. The camera used in photographing the excursions of the string was a 12 cm. camera, also made by the Cambridge Instrument Co.

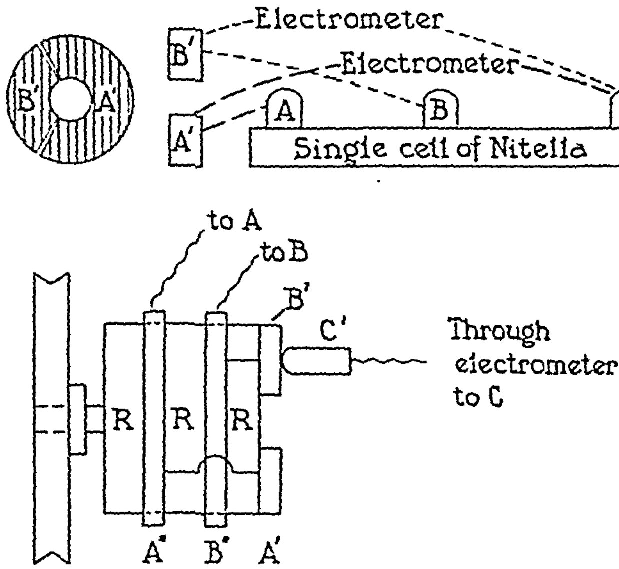

FIG. 5. Diagram of the rotary switch. The two brass segments A' and B' are insulated from each other and are connected to the rings A'' and B'' fastened to the hard rubber cylinder R: as they revolve they come alternately in contact with the brush C' which is connected through the electrometer to the point C on the cell. When B' is in contact with the brush the circuit is closed through B', C', B, and C; when A' is in contact with the brush the circuit is closed through A', C', A, and C. As the contact with A' is twice as long as with B' the dash is twice as long on the record, as indicated by the broken lines connecting A and B to the rotary switch.

The switch, which connects the filament to either the grid or one of the calomel electrodes, must not open one circuit before it closes the other. A battery, marked "*C* battery" in the figure, is inserted to polarize the grid negatively. The voltage of the *C* battery must be chosen from a study of the static characteristic curves of the tube being used, under the same conditions of plate and filament voltage as are used in the circuit. Under these conditions no appreciable current flows

between grid and filament. In order to obtain reversibility[13] and good proportionality, it is necessary to operate the tube on the straight part of the curve obtained by plotting the grid voltage against the plate current.

The resistance R_G is a grid leak which is inserted during certain types of experiments to protect the string in case an open circuit should occur at the *Nitella* cell. The nearer the polarizing potential of the C battery approaches the free grid potential the less the necessity for the grid leak which may therefore have a very high resistance or be omitted altogether.

If a grid leak is employed the observed values of the potential difference are lowered. The amount of this lowering was ascertained by making measurements with and without the grid leak: the latter agreed with simultaneous readings made with the Compton electrometer. These measurements were made under all the experimental conditions which alter the resistance of the *Nitella* cell (such as change of solutions, killing part or all of the cell) and a correction factor was applied to the observed values. A convenient way to ascertain the correct value is to make calibrations (by means of the potentiometer P') as often as there is any reason to suppose that the resistance changes.

In order to switch from one point to another on the cell it was necessary to employ a rotary switch such as that shown in Fig. 5. In its simplest form this consists of two brass rings (A'' and B'') mounted on the periphery of a hard rubber cylinder (R). Each ring is connected by an electrical conductor to a brass segment mounted on one end of the cylinder, one segment (A') consisting of an arc of 240°, another (B') of an arc of 120°. This gives a ratio of 1:2. The segments are insulated from each other, and a brush (C') makes contact with them as the whole unit revolves. The rotary switch is made to revolve at a rate which is proportional to the rate at which the bromide paper passes through the camera. The point A on the cell is connected to A' so that when the brush is in contact with A' the circuit is completed from A to C through the electrometer: when the brush is in contact with B' the circuit runs from B to C through the electrometer but as this connection lasts only half as long as in the case of A the dash on the record is only half as long, as is indicated by the broken lines in the figure.

As the brush passes from one segment to another it remains for a very small fraction of a second in contact with both segments so that for this interval both A and B are connected to C. It might seem that this would allow A to discharge to B (or *vice versa*) but it is evident that this does not affect the results for we get exactly the same values when we do not employ the rotary switch.

When connection is made to more points on the cell the rotary switch has a corresponding number of segments.

The apparatus was adjusted by applying a known potential to the grid and filament circuit, and varying the tension of the string and the resistance in series

[13] The reversibility was tested during each experiment and unless it was satisfactory the experiment was rejected.

with the string, until the desired sensitivity was obtained. The known *potential* was obtained from a Leeds and Northrup potentiometer which was standardized against a Weston standard cell. The entire electrical apparatus and the wire used in all connections was electrostatically shielded. The box containing the vacuum tube and accessory apparatus was suspended from the ceiling (by means of springs and rubber) to avoid mechanical shocks. The string galvanometer was mounted on sponge rubber.

As has been stated, the plan of the experiments was to reduce the potential difference at one point approximately to zero[14] by killing the protoplasm at that point. It seemed probable that this could be done since in experiments on *Valonia macrophysa* (arranged as in

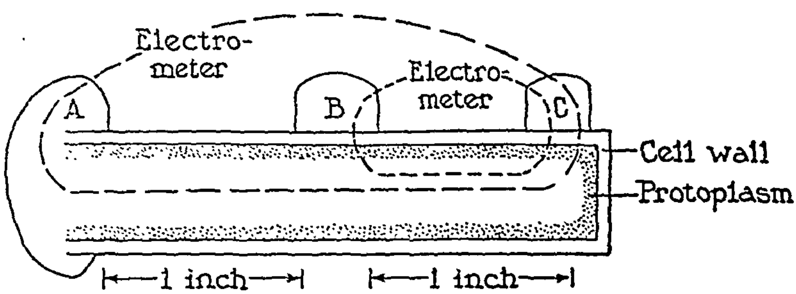

FIG. 6. Diagram to show the circuits in a cell cut open at *A*. The circuit going through *A* passes only once through protoplasm: that going through *B* passes twice through protoplasm. In consequence *A* and *B* do not as a rule act alike. (The thickness of the cell wall and of the protoplasm (which is only a few microns) is greatly exaggerated in proportion to the diameter of the cell.)

Fig. 1) we find, on leading off from the interior of the capillary to sap in contact with the outside of the cell a potential difference of about 14.5 millivolts; but when we apply sap saturated with chloroform the potential difference falls nearly or quite to zero.

In order to test this idea in the case of *Nitella* an experiment was arranged as shown in Fig. 2: sap saturated with chloroform was applied at *A* and *C* or at *A*, *B*, and *C* after which the cell was cut open at *A*.[15] If the cut opens the protoplasm in the manner shown in Fig.

[14] By this is meant a low value not exceeding 2 millivolts.

[15] If *A* and *C* were alive cutting at *A* would permanently change the potential difference. This is not the case when they have been completely killed by chloroform.

6 the circuit from A to C will pass only once through the protoplasm and practically all the electromotive force will be due to the dead protoplasm at C (when C is killed sap comes out so that the cell wall is in contact with sap on both sides and will in consequence produce no electromotive force). Under these circumstances the average value was close to zero.

Since the circuit from B to C passes through protoplasm at two places it may be expected to act differently from the circuit passing from A to C, as long as B is not completely killed, but when B is dead both circuits should act alike when tested by applying toxic agents or by changing the composition or the concentration of the solutions without involving toxic action (as described below). This was found to be the case.

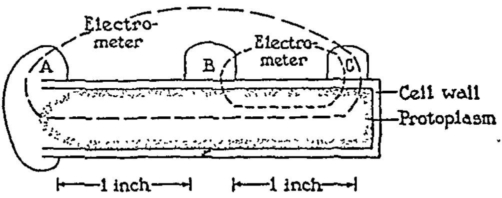

Fig. 7. Like Fig. 6 except that the cut has brought the protoplasm together at A.

In cutting A it may happen that the protoplasm is brought together in the fashion shown in Fig. 7, as the result of which (when subjected to the tests mentioned below) the circuit from A to C acts for a time as if the protoplasm at A were not completely dead. As long as this condition lasts we cannot obtain the correct value of C. But when the tests show that the protoplasm at A is completely killed we find that the observed value in the circuit A to C falls almost or quite to zero.

It would therefore seem that when a spot is completely killed by chloroform its mean value is not far from zero and hence by leading off to another spot which is far enough away to remain uninjured we should be able to measure the normal potential difference across the

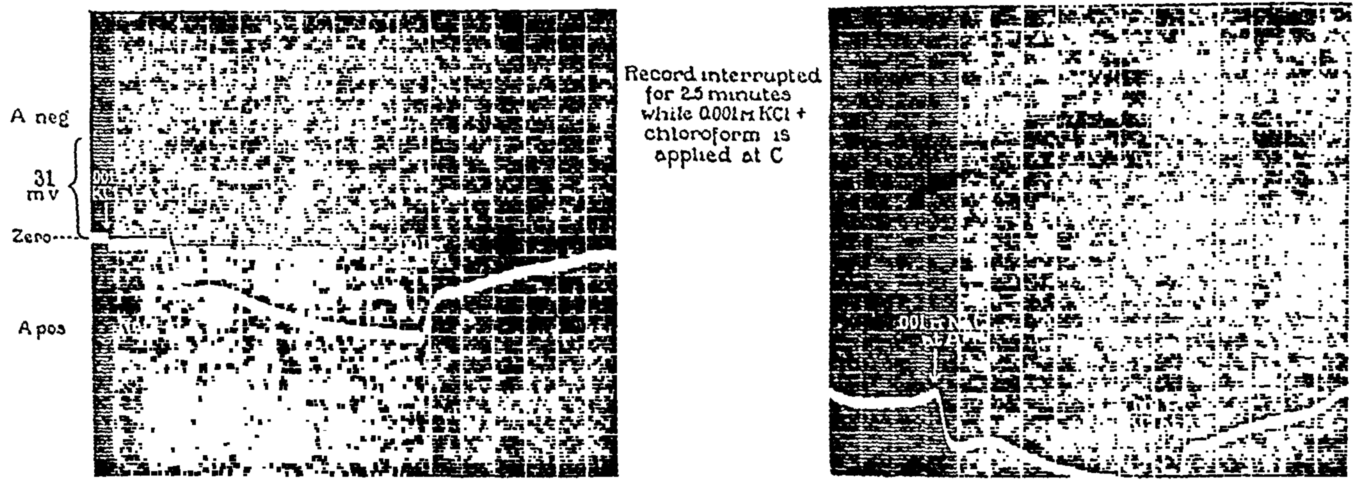

FIG. 8. Photographic record of an experiment arranged as in Fig. 2 (omitting *B*) with flowing junctions at *A* and *C*. The curve shows the state of *A* with reference to *C* (*i.e.* when curve *A* is positive the point *A* is positive to *C*). At the beginning of the record 0.001 M KCl is applied at *A* and *C*: then 0.001 M NaCl is applied at *A* causing a positive drop which disappears when 0.001 M KCl is again applied at *A*. The record is then interrupted for 2.5 minutes while 0.001 M KCl saturated with chloroform is applied at *C*: This results in a positive drop because the killing of *C* reduces its electromotive force nearly to zero so that what remains is due to *A* which is positive because in contact with 0.001 M KCl. A new application of 0.001 M NaCl at *A* produces the same positive drop as before, thus indicating that there has been little or no injury: on applying 0.001 M KCl this disappears, as before. The vertical lines represent 5-second intervals. Selected as typical from over 60 experiments.

protoplasm at the latter spot. We can arrange an experiment as in Fig. 2 and kill with chloroform at *C* and then apply various tests to discover whether *A* remains normal. All of these tests agree in showing that this is the case for some time after the chloroform is applied at *C* (but it is of course possible that there may be injury which is not detected by these tests). The tests are as follows:

1. The appearance under the microscope. A very slight rounding up of the chloroplasts is the first sign of injury.

2. The application of certain innocuous solutions which produce very different effects on normal and dead protoplasm. When, for example, we substitute 0.001 M NaCl for 0.001 M KCl normal protoplasm gives a characteristic response (becoming more positive) which is lacking in dead protoplasm and which is not affected by the cell wall (as shown by experiments on dead cells). This is illustrated by the photographic record in Fig. 8 which shows that the substitution of 0.001 M NaCl for 0.001 M KCl produced a positive drop which disappeared when 0.001 M KCl was again applied at *A*. Chloroform was then applied at *C* and about 2.75 minutes later 0.001 M NaCl was again applied at *A*. It produced the same drop as before, indicating that no injury had occurred[16] (it does not follow, of course, that this would apply in every case and to all kinds of material).

Exactly the same sort of evidence is secured by using two concentrations of the same salt (*e.g.* by changing from 0.01 M KCl to 0.001 M KCl) although in this case there is some complication due to the effect on the cell wall.

3. The application of toxic solutions produces very different effects on living and dead protoplasm. Thus we find that for some time after killing *A* with chloroform the application of chloroform to *C* produces the same sort of curve as at *A*, but if we wait too long the character of the curve changes. (If we cut the cell open at *A* instead of applying chloroform, *C* promptly shows signs of injury. This cannot be discussed in detail here but will be fully described in later papers.)

[16] In this case sap escapes at *C* when the protoplasm is killed and comes in contact with the cell wall. This would affect the observed potential difference (since the cell wall at *A* is in contact with 0.001 M KCl or NaCl) were it not for the fact that the flowing junction reduces the effect to negligible proportions.

4. If any injurious effects produced by chloroform at *A* should spread along the cell to *C* the potential difference between *A* and *C* would change (this change would as a rule be wholly due to *C* since *A* would usually remain constant after death). To test this an experiment was arranged as in Fig. 2 (with *B* omitted) with sap[17] at *A* and *C*. Sap saturated with chloroform[18] was applied at *A*. The records show that as a rule the potential difference between *A* and *C* remained

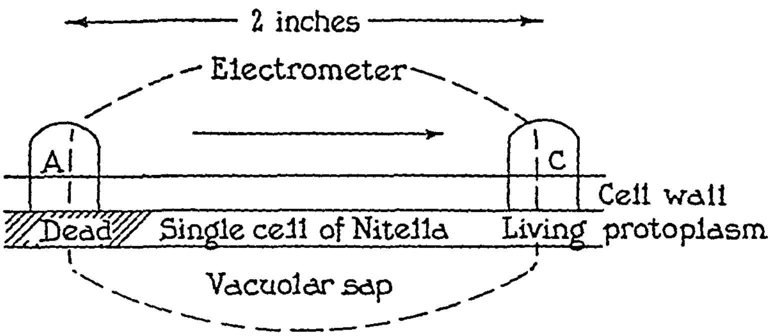

FIG. 9. Diagram of the circuit in a cell of *Nitella* with cell sap at *A* and *C*. The protoplasm at *A* has been killed by chloroform thus reducing the potential difference at *A* approximately to zero so that the electromotive force of the circuit is practically all due to the living protoplasm at *C*. The arrow shows the direction in which the positive current tends to flow through the electrometer.

constant for some minutes, after the subsidence of the disturbance produced by the application of chloroform at *A*.

The fact that the potential difference between *A* and *C* remains constant indicates that no injury has spread along the cell from *A* to *C*. We may therefore regard *C* as unchanged during the period of the experiment. On the other hand, when the changes due to the appli-

[17] This was obtained by cutting off the ends of living cells (the surface being lightly dried with filter paper) and causing the sap to flow out by gentle pressure. It was used at once as it alters on standing and becomes unfit for use in such experiments as these. Wads of purified cotton or filter paper were soaked in the sap and applied to *A* and *C*.

[18] The fact that chloroform causes a rapid change has been shown by one of us in a different way (exosmosis). *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 709. Chloroform has the advantage of producing scarcely any change in electrolyte content or osmotic pressure when sufficient is added to saturate the solution.

cation of chloroform at A have subsided and the curve has flattened out we may regard A as dead since we find little or no change in potential difference when we apply to A the tests mentioned above: these would cause a considerable change of potential if A were alive, or only partly dead. When these solutions are applied to C, not too long after the death of A, they give the expected change of potential difference, indicating that C has remained normal.

The experiments indicate that when the protoplasm at A is completely killed by sap saturated with chloroform its electromotive force falls approximately to zero and the electromotive force which remains[19] is due to the living protoplasm[20] at C which remains for some time in normal condition.[21] Measurements were therefore made by ascertaining the potential difference between A and C as soon as the changes due to the application of chloroform at A had subsided and the curve had flattened out.

With sap at A and C we find that A is positive to C, that is, the positive current tends to flow through the electrometer in the direction shown by the arrow in Fig. 9. This indicates that when sap is applied to the outside of the cell the inner surface of the protoplasm at C is positive to the outer surface so that if we could substitute *Nitella* for *Valonia* in Fig. 1 the positive current would tend to flow in the same direction as in *Valonia* (as shown by the arrow). The potential difference in *Valonia* (with *Valonia* sap applied to the exterior) is about 14.5 millivolts and the corresponding value in *Nitella*

[19] There can be no cell wall effect, since the solutions at A and C are practically identical in respect to electrolytes and experiments show that these solutions give no potential difference on dead cells.

[20] The application of sap to the outside of the cell produces no injurious effect in these brief experiments, as is shown by the fact that when an experiment is arranged as in Fig. 2, with tap water at A and sap at C, the potential difference remains unchanged for a period considerably longer than the duration of the experiments described in this paper. In longer experiments the sap may have some injurious action.

[21] The length of this time varies with the material but can always be tested in the manner described above and in no case were experimental results regarded as valid unless the measurements were taken before there appeared to be any sign of injury.

(with *Nitella* sap applied to the exterior) is about 15.9 millivolts.[22]

We thus arrive at the conclusion that in *Nitella*, as in *Valonia*, the protoplasm is asymmetrical. If, for example, we suppose it to be made up of layers, X, W, and Y, as suggested in a former paper,[1] we should have the chain[23]

$$\text{(external) sap} \mid X \mid W \mid Y \mid \text{sap (internal)}$$

If X and Y were identical we should have no potential difference but since this is not the case we must suppose that they are unlike.

It is therefore evident that asymmetric protoplasm is not confined to *Valonia* but that it exists in *Nitella* also and it is possible that it may be a general phenomenon. In that case there can be little doubt that it is of fundamental significance. In all probability this phenomenon is closely connected with the differences in selective permeability between the inner and outer protoplasmic surfaces that have been previously pointed out.[1] It is in harmony with the fact that in *Nitella* and in *Valonia* the two surfaces appear to react differently to cell sap which seems to be injurious to the outer surface but not to the inner. But it is of course possible that even if X and Y were alike the application of sap to the outside might cause injury by changing the normal potential difference across the protoplasm or in some other way disturbing the normal relations.

It would be an interesting problem to determine by what mechanism these two layers are formed in a film of protoplasm which is only a few microns in thickness. The idea that the surface layer of the protoplasm is produced by the migration into the surface of substances which reduce surface tension would lead us to expect that both the inner and outer surfaces would be alike. That they are not alike is

[22] This is the average of 29 experiments, the probable error of the mean being $\pm$ 0.744 millivolts or 4.65 per cent of the mean. A few of these experiments were made with artificial sap (80 parts 0.05 M KCl + 20 parts 0.05 M NaCl) which gives the same results as natural sap.

This value might be higher if there were no short circuits in the cell: these have been discussed in a previous article.[1]

[23] The cell wall is omitted since it does not appear to affect the results here described.

indicated by the results described above as well as by other considerations such as the fact that a cellulose wall is deposited at the outer surface, but not at the inner.[24] It might be supposed that this is due to the fact that the two surfaces are not under the same conditions since they are in contact with different solutions. If this were the case we should expect that a cell bathed in its own sap could not secrete a cell wall. Unfortunately, this experiment cannot be carried out because the sap is too toxic.

In addition to the inner and outer surfaces of the protoplasm which seem to be unlike there may be a variety of internal surfaces. It should be remembered that in both animals and plants there are cases where different kinds of vacuoles exist in the same cell and it is possible that each of the different kinds of inclusions in the cytoplasm are in contact with a different kind of protoplasmic surface. In addition to these there are the surfaces of nuclei and of plastids as well as of mitochondria. There may be considerable diversity among these surfaces in respect to permeability and electrical properties.

The fact that the inner and outer surfaces of the protoplasm are unlike might be regarded as analogous to the fact that the two ends of a cell are in many cases unlike. If in the latter case we may speak of the longitudinal polarity of the cell, we might in the former speak with equal propriety of radial polarity, meaning that in one case the unlike elements of the protoplasm are arranged along a longitudinal line and in the other along the radii of the cross-section of the cell. The nature of both kinds[25] of polarity deserves careful investigation.

SUMMARY.

Using multinucleate cells of *Nitella* 2 or 3 inches in length it is possible to kill one end with chloroform without producing at the other any immediate alteration which can be detected by our present methods.

[24] It is, of course, quite possible that a very thin film of cellulose or some other substance is secreted on the inner surface, comparable to the thin films found on the surface of many protozoa, but this is evidently not the same thing as building up such a cellulose wall as we find at the external surface.

[25] In many cases both are present simultaneously in *Nitella*.

When a spot in external contact with sap is killed its potential difference falls approximately to zero and it is therefore possible to measure the potential difference across the protoplasm at any desired point merely by leading off from that point to the one where the protoplasm has been killed.

The results indicate that the inner and outer protoplasmic surfaces differ, for when both surfaces are in contact with the same solution (cell sap) there is an electromotive force of about 15.9 millivolts, the inner surface being positive to the outer (*i.e.* the positive current tends to flow from the inner surface through the electrometer to the outer surface). The situation resembles that in *Valonia* where the corresponding value (with *Valonia* sap applied to the outside) has been reported as about 14.5 millivolt[26] (the inner surface being positive to the outer). It would seem appropriate to designate this as radial polarity.

[26]But later measurements indicate that this value is too low.

TEMPERATURE CHARACTERISTICS FOR THE GROWTH OF THE SPORANGIOPHORES OF PHYCOMYCES.

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I.

The growth of an organism is surely a complex process, but it is known that its rate may be regulated by changing the temperature and, often, the light intensity. Studies on the temperature relations of growth are complicated, however, by the usual sigmoid character of the growth curve, which makes difficult a comparison of the rates under different circumstances (Crozier, 1926–27; Brown, 1927–28). For this reason material was sought which should have a simple, measurable form and a constant growth rate. This was found in the sporangiophores of the fungus *Phycomyces*, where over a period of 6 to 12 hours growth is practically a linear function of the time, and is identical with the elongation of the sporangiophore (which remains isodiametric throughout). Furthermore, each sporangiophore, and theoretically the whole fungus culture, is without cross-walls, thus eliminating the intricacies resulting from a consideration of growth as a problem in the statistics of cell number.

The following experiments deal with the relation between temperature and the growth of *Phycomyces;* work is in progress concerning light, which is simultaneously a regulating factor.

II.

Cultures of *Phycomyces blakesleeanus* ("+" strain)[1] were grown in short glass vials on a sterile medium of bread wet with prune juice. When a mass of young sporangiophores was formed, the culture was transferred to an ice chest at 4°C.,

[1] I wish to thank Professor A. F. Blakeslee for his kindness in supplying these cultures.

where growth was slow, and kept there until needed. Since all the temperature effects found have been completely reversible after a short interval, there can be no question here of thermal adaptation.

For use, a culture vial was placed in a little water at the bottom of a tall glass cell with plane sides and top, nearly submerged in a large, rectangular water

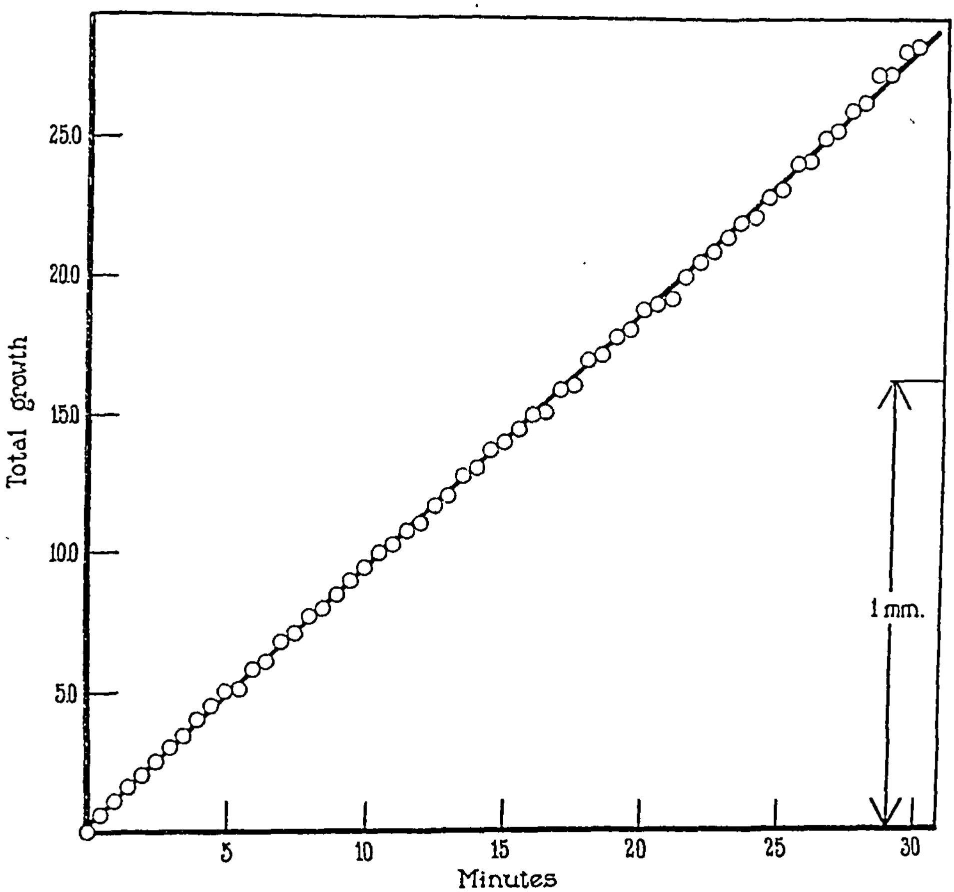

FIG. 1. The elongation of a typical sporangiophore of *Phycomyces* with time. Deviation from a straight line relationship is imperceptible over much greater periods of time. Ordinate values are in arbitrary units from the ocular micrometer scale.

thermostat similar to that described by Crozier and Stier (1926–27, *b*), and capable of being regulated to within $\pm$ 0.004°C. Two parallel pieces of plate glass were let into opposite sides of the thermostat to serve as windows for lateral observation of the immersed cell and its contents, and a small dark room was built around the whole apparatus.

Observation of the fungus was made by means of a long-focus horizontal

microscope, with vertical and horizontal adjustments, which projected into the dark room opposite one of the windows of the thermostat. Through the other window came a faint red light which was flashed on at intervals to give a silhouette of the sporangiophore against an ocular micrometer scale. The vertically growing sporangiophores, which have an exceedingly low threshold of phototropic sensitivity, were continuously oriented by a constant intensity of light admitted from above through a tube containing heat filters and diffusing screens.

At least 15 minutes were allowed for the establishment of thermal equilibrium after each temperature change. Readings of the position of the sporangium on

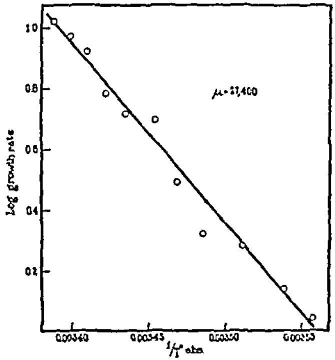

FIG. 2. The rate of growth of one sporangiophore of *Phycomyces* as a function of temperature. The apparent spiraling of the points about the line in this and other plots is random and without significance, as is shown by other series of observations, except that greater variability in rate frequently appears at "critical temperatures."

the micrometer scale were usually made at intervals of 1 minute; with low temperatures, less frequently.

The rate of growth at a particular temperature is determined by plotting the micrometer ocular readings against time. A minimum of ten points is taken, usually more, and the best straight line drawn through them. This is always justified, unless irreversible changes are taking place, since the practical constancy of the growth rate of *Phycomyces* sporangiophores over long periods of time is well established (Fig. 1, *cf.* Blaauw, 1914; Graser, 1919). The slope of the line is taken as the rate.

III.

When for a particular sporangiophore the logarithm of the growth rate is plotted against the reciprocal of the absolute temperature, the points are typically found to have a linear sequence, fluctuating about a mean straight line. Characteristic plots of this sort are given in

TABLE I.

μ	Temperature range	Intensity of vertical illumination
		foot candles
9,800	19°–26°	Low
10,000	20°–26°	Low
11,500	7°–29°	2.5
11,900*	16°–28°	2.5
12,000–13,000	12°–23°	5.7
16,500	21°–25°	2.5
19,500	19°–25°	Low
20,100*	7°–16°	2.5
25,000	7°–17°	2.5
26,300	9°–21°	2.5
27,400	8°–22°	2.5
33,400	9°–13°	2.5

*Same experiment. Shift in rate as well as in increment around 16°.

Figs. 2 and 3. In the Arrhenius equation relating the velocity of chemical reaction and temperature,

$$\frac{\text{Rate at } T_2°}{\text{Rate at } T_1°} = e^{\frac{\mu}{2}\left(\frac{1}{T_1°} - \frac{1}{T_2°}\right)}$$

the significance of the constant μ in relation to the rates of vital processes has been discussed in detail by Crozier (1924; 1925–26, *b*; and other papers). In Table I are given all the values of μ obtained, these being calculated from plots similar to those in Figs. 2 and 3. It is evident that the values group themselves about modes which are in the vicinity of 11,000, 16,000, 20,000, 26,000, and 33,000 calories.

The magnitude of μ is not correlated with the absolute growth rate. These numbers agree strikingly with those listed by Crozier (1925–26, *b*) for a great variety of biological processes. The temperature characteristics computed for the *early* growth of *Rhizopus* hyphæ are 16,800 and 15,700; for *Gibberella* colonies, 11,100 (Crozier, 1926–27).

While some sporangiophores may follow a single temperature characteristic from 7°C., below which temperature difficulties in experimentation become great, to around 26°C., which is the region of

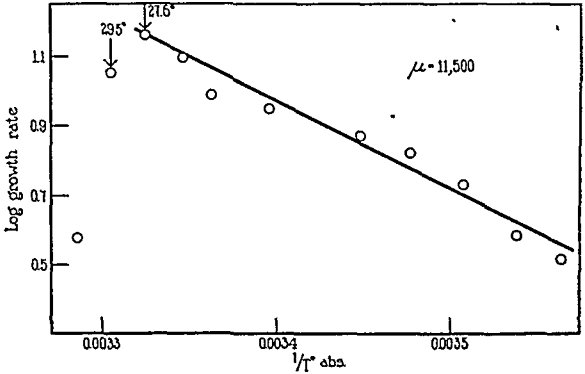

FIG. 3. The rate of growth of a *Phycomyces* sporangiophore as a function of temperature. A comparable value of μ (11,100) has been computed for the growth of *Gibberella* colonies (Crozier, 1926–27). Thermal destruction is seen here near 27°, but precise estimation of its locus is difficult because of a lag in the response of the sporangiophore. In passing from the region of thermal destruction to a lower temperature there is prolonged hysteresis.

incipient thermal destruction, other individuals may show "breaks" or changes in absolute rate of growth, or both simultaneously (as in Fig. 4; *cf.* Crozier and Stier, 1926–27, *a*; 1926–27, *b*). The specific theory of abrupt changes of thermal increment as related to possible shifts in control in a series of catenary chemical reactions has been treated by Crozier (1924, etc.), as well as the significance of the "critical temperatures" at which irregularities or innovations may appear (Crozier,

1925–26, *a*). There is considerable evidence that with *Phycomyces* the region of 15° is a critical one, and in many of these temperature plots a slight absolute falling off in rate without definite change of μ is observed near 20°.

It is important to note that any process of averaging the results on different sporangiophores, either from the same or from different cultures, as Graser (1919) did, would certainly lead to chaotic results, since both absolute rate and thermal increment may vary from one

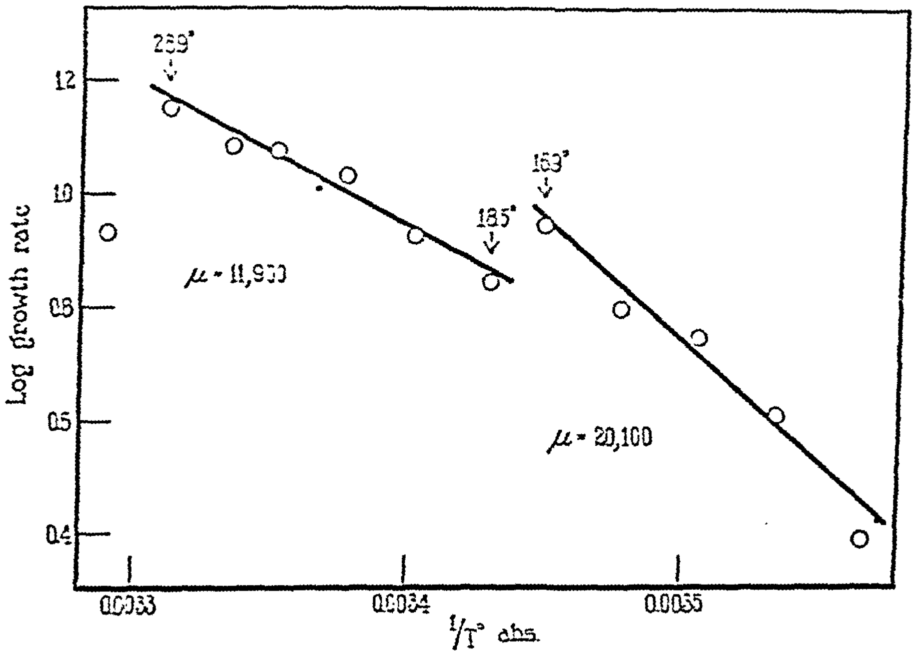

FIG. 4. The rate of growth of a sporangiophore of *Phycomyces* as related to temperature. This experiment was run from a low temperature to the highest, and was not checked by lowering the temperature. It is possible that the "break" is not reversible, a circumstance which has been found with *Notonecta* "hearts" (Crozier and Stier, 1926–27, *a*). Similar plots have been obtained with embryonic *Limulus* hearts (Crozier and Stier, 1926–27, *b*).

individual to another. There is no reason, for example, to suppose uniformity of conditions at the bases of two sporangiophores, because there must be a changing gradient of foodstuffs and split products throughout the mycelial mat. An attempt was early made to eliminate this complication through the use of a sterile, flowing medium, but under these circumstances the fungus does not fructify. The con-

siderable variety of temperature characteristics that has been found agrees well with the known complexity of culture conditions, and there is no reason to suppose that all of the possible values have been encountered.

IV.

The range of values of critical thermal increments for the growth of *Phycomyces* is of interest as concerns the general theory of growth, in showing that there is not *one* temperature coefficient for this process—*a* "temperature characteristic for growth"—and, furthermore, in regard to the possible identification of the values found with a variety of specific catalysts found to control the rates of vital processes.

SUMMARY.

1. With constant temperature and light intensity, the rate of elongation of a sporangiophore of *Phycomyces* is constant for many hours.

2. With constant light intensity, values of μ, the "critical thermal increment," have been computed for the elongation of different sporangiophores. These values group themselves closely about the modes 11,000, 16,000, 20,000, 26,000, and 33,000 calories. Sporangiophores from the same culture need not have similar increments.

3. The significance of these results for studies of growth is discussed.

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THE EFFECT OF TEMPERATURE ON THE MECHANICAL ACTIVITY OF THE GILLS OF THE OYSTER (OSTREA VIRGINICA Gm.).

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INTRODUCTION.

The gill of an oyster is a complex, ciliated organ that takes part in three important functions of the organism: respiration, feeding, and excretion. One of its most noticeable activities consists in producing a strong current of water, which passes through numerous branchial chambers and insures the exchange of gases between the tissues of the organism and the surrounding medium. The material suspended in water and brought in with the current constitutes the food of the oyster. It settles on the surface of the gill and, after being entangled in the mucus excreted by numerous gland cells, is pushed by the ciliary epithelium toward the distal edges of the gill laminæ and is conveyed to the labial palps, where it is either rejected or enters into the digestive tract. When the oyster is not feeding and keeps its valves closed, the gland cells of the gills continue to excrete mucus which accumulates in a large quantity on the surface of the gill and is discharged into water at the first opportunity. The structure of the lamellibranchiate gill has been the object of numerous investigations, and for a detailed anatomical and histological description the reader is referred to the works of Ridewood (1903), Janssens (1893), Peck (1877), and Kellog (1892). It is necessary, however, for the purpose of the present paper, to state that the gill of the oyster can be compared to a very fine and complex sieve, the holes of which are represented by the water pores; the water is taken in by the whole surface of the gills and is driven through a system of tubes into one exhalant chamber. It leaves the gills as a single outgoing stream, which can be observed

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easily when the oyster is feeding. Through the water pores and tubes there is direct communication between the inside and outside of the gill, and the flow of water in one direction is due exclusively to the rhythmical beats of the lateral cilia (Fig. 1.) These facts have an important bearing on the discussion of the experimental data.

Method.

The method employed in this work consists in measuring the rate of flow of water produced by the gills at different temperatures. For this purpose the

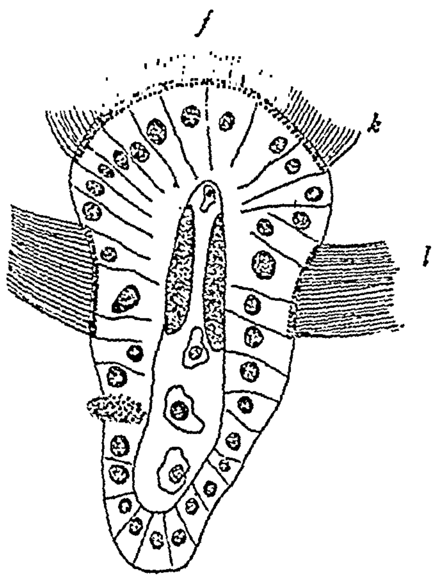

FIG. 1. Filament of the oyster gill; transverse section. Camera lucida drawing. *f*, frontal cilia; *k*, fronto-lateral cilia; *l*, lateral cilia.

valves of the oyster are forced apart and a glass rod is placed between them to prevent their closing (Fig. 2); a rubber tube, *A*, from 6 to 7 mm. in diameter, is inserted into the cloaca and made fast by packing all the spaces around it with cotton. The exhalent current passes through the tube; leakage, if any, can be noticed easily by adding a few drops of carmine suspension and watching the produced currents. The oyster is then placed in a glass tray of about 4 liters capacity. The end of the rubber tube, *A*, is connected to a tube, *B*, the upper end of which is attached to a funnel, *F*, filled with a fine suspension of carmine in sea water. The third end of the tube, *B*, is connected with a graduated glass tube, *C*, 6 mm. in diameter and 17 cm. long. Releasing the clamp, *D*, a very small amount of carmine is allowed to enter the tube, *C*, where it forms a distinct cone,

moving inside the tube. The rate of movement of the apex of the cone is measured by recording with a stop-watch the time during which it passes from 0 to the 15 cm. mark. The temperature of the water is changed by using either an electric hot point immersion heater or a battery of jars filled with a freezing mixture. The water in the tray is agitated by an electric stirrer and is aerated. If necessary, the tray is placed in a water jacket with a mixture of salt and crushed ice packed between the walls. Readings are made after the oyster has been left for 15 minutes at a given constant temperature (possible fluctuations = ±0.5°C.). At every given temperature from 10 to 20 readings are made, from which the arithmetic mean is computed. From the figures thus obtained and representing the velocities of the current at the center of the tube, the discharge of water (in

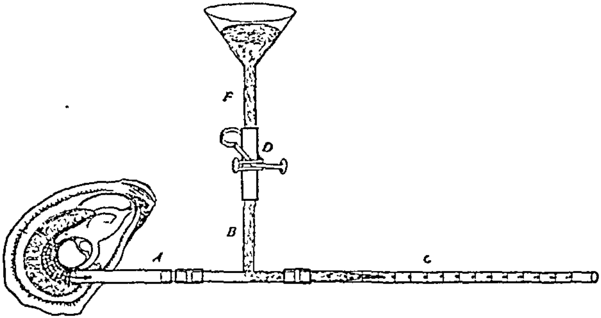

FIG. 2. Method of measuring the rate of flow produced by the gills. The *oyster is shown with the left valve and mantel removed. Explanation in text.*

liters per hour), and the work performed in propelling it through a given tube can be computed.

Inasmuch as a distinct cone of carmine suspension is visible, it may be assumed that in this case we have a viscous flow or "stream line," to which the Poiseuille's formula:

$$S = \frac{D^2 \Delta p}{16 \mu l} \qquad (1)$$

or

$$\Delta p = \frac{16 \mu l S}{D^2} \qquad (2)$$

is applicable. In his formula S = speed at the axis of the tube in cm./sec.; D = diameter and l = length of tube in cm.; Δp = pressure drop between the two marks in dynes/ cm.^{2}; μ = viscosity in poises (C. G. S. unit).

As the mean velocity (S_M) of the whole cross-sectional area of the tube is one-half of the velocity at the axis,[1]

$$S_M = \frac{S}{2} \tag{3}$$

the rate of discharge, V, in cm.^{3}/sec.,

$$V = \frac{\pi}{4} D^2 \frac{S}{2} = \frac{\pi D^4 \Delta p}{128 \mu l} \tag{4}$$

The rate of doing work W (in ergs/ sec.) is equal to the product of discharge, V, by drop in pressure, p.

$$W = V \Delta p \tag{5}$$

Substituting the values of V and p (formulæ (2) and (4)), we arrive at a simple expression,

$$W = 2\pi l \mu S^2 \tag{6}$$

Applying formulæ (4) and (6), both the discharge and the rate of doing work can be computed. In an analysis of the effect of temperature on the activity of the ciliated epithelium, the latter figures have an obvious advantage because they eliminate the error introduced by the changes in the viscosity of sea water. In computing the rate of doing work, the values of the viscosity of sea water were obtained by interpolation from the data given by Krümmel and Ruppin (1905). The salinity of water in the present experiments varied from 28.0 to 31.6 parts per thousand.

In computing the work produced by the gills, the length of the tube, l, was taken as equalling 15 cm. As all three tubes (Fig. 2) are approximately of the same diameter, it might be possible to use the entire length from the oyster to the end of the tube C; but this probably would be less accurate on account of the unknown amount of loss of head or resistance, due to rubber and L-tube connections. Thus, the figures computed from the experimental data represent the work that is expended in producing a stream between the 0 and 15 cm. marks in the glass tube C. The changes in the dimensions of the tube, due to the thermal expansion of glass, are so small that the possible inaccuracy due to this source is much less than the experimental error.

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[1] See Gibson, 1925, p. 63.

Comparison with the Other Methods.

Attempts to measure the energy of the ciliary motion have been made by many investigators who applied methods entirely different from that adopted in the present investigation (Calliburcès (1858), Bowditch (1876), Engelmann (1879), Jensen (1893), Vignon (1901), Orton (1912), Merton (1923), Gray (1923–24), Yonge (1926)). Most of these experiments were made with a small piece of epithelium cut away from the organism and kept for a considerable period of time in sea water or in balanced salt solution, where it undoubtedly was undergoing irreversible changes.

The present method eliminates the main difficulties encountered in previous experiments. First, the organism is kept intact and the gills work under normal conditions; second, there is no stimulation of the ciliary epithelium caused by contact with foreign bodies.

The experiments were carried out during the summers of 1925 and 1926 and in the winter of 1927 at the United States Fisheries Biological Station at Woods Hole, Massachusetts. The oysters used in the experiments were received from Long Island Sound, Wellfleet Harbor, Massachusetts, and Chesapeake Bay, Virginia. There was no differences in the behavior of oyster from these three localities.

The author desires to express his gratitude to Dr. E. Buckingham, United States Bureau of Standards for the advice in hydrodynamical problems involved in the present investigation.

Effect of Temperature on the Rate of Doing Work.

The beat of the ciliary cell has two distinct phases (*a*) a very rapid forward or effective stroke, and (*b*) a slow backward or regressive stroke. The energy expended during each phase can be computed by using the formulæ given by Weiss (1909) and Gellhorn (1925). When the ciliary motion proceeds in one plane, the cilium is straight and the velocity is constant, the resistance (p) to a middle point of the cilium lashing in the water is proportional to the square of velocity (v)

$$p = kv^2 \tag{7}$$

where k is a constant. The energy, E, expended during one phase is

$$E = ps = ksv^2 \tag{8}$$

where s is the amplitude. The work performed by the cilium in time, t, is

$$W = \frac{ps}{t} = kv^3 \tag{9}$$

The ability of the ciliated cells to transport the particles or produce a current of water depends on the ratio between the progressive and regressive phases. If for instance the velocity of the progressive phase, as has been shown by Kraft (1890), is five or six times greater than that of the regressive stroke, the work performed by the first one is 125 or 216 times greater than that of the latter. It is obvious that even small changes in the ratio between the velocities of two strokes result in marked changes in the effective work of the cilium.

Besides the ratio of the velocities of the progressive and regressive strokes, the efficiency of the ciliated epithelium depends on three other factors: amplitude of the strokes, their frequency, and the rhythm of the ciliary motion along the whole surface of the organ. One of the most remarkable features of the ciliary mechanism is the metachronical rhythm which is due to the fact that any individual cell begins its progressive stroke slightly before the cell situated on one side of it and after that located on the other side. In a complex structure like the gill of an oyster the current running from the epibranchial chambers to the outside is produced by the thousands of lateral cells that beat with a definite succession and cause the increase of pressure inside the gill cavity. The maintenance of a given pressure depends on a definite rhythm of strokes along all the filaments of the gill. As soon as in some of the filaments the latter slows down there occurs a leakage that causes a drop in pressure resulting in a decrease or a complete stoppage of the current. In this manner, even a small disturbance in the rhythm of beats in some of the filaments may cause considerable fluctuation in the velocity of the outgoing current.

Physical and chemical changes in the surrounding medium have a marked effect on the behavior of the ciliated cells. Gray (1923–24) has shown that not only temperature but changes in the pH value, O_2, and CO_2 content affect the ciliary activity. In experimental work it is necessary, therefore, to eliminate the effect of all the variables except that which is studied. In the present experiments the salinity of the water, its oxygen content, and the pH value were kept constant. There are two factors, however, the control of which presented certain difficulties and which may be responsible for considerable fluctuation of the experimental data. In some of the oysters, especially in those that have been exposed for a long time to a low

temperature, the gills were covered with a thick layer of mucus which blocked a free passage of water through the pores. After the outside and the inside of the branchial chambers were washed out with sea water, the current became steady. Mechanical stimulation represents another factor that may affect the velocity of the current. Whenever the oyster attached to the apparatus was disturbed, it invariably showed a change in the rate of flow, frequently stopping the current entirely but coming back to normality in a few minutes. The following record of one of the experiments illustrates this fact very clearly.

Experiment 62, August 10, 1926.

Speed at the axis of the tube	Temperature	Time
cm./sec.	*°C*	*a m.*
1.1	14.2	11.03
1.1	14.2	11.04
1.1	14.2	11.05
1.1	14.2	11.06
1.2	14.2	11.07*
0.6	14.2	11.08
0.6	14.2	11.09
1.1	14.3	11.10
1.0	14.3	11.11
1.0	14.3	11.12

* Oyster disturbed.

It is very probable that these fluctuations are due to the contraction of the branchial chambers or plicæ, caused by mechanical stimulus. In the experiments described below the precaution was taken to avoid mechanical stimulation; and in case the oyster was disturbed by accident, it was left for 10 minutes before the next readings were made.

The experimental data obtained during the summers of 1925 and 1926 consist of 167 determinations of the rate of doing work of 15 oysters subjected to different temperatures, ranging from 5° to 45°C. Each determination is a mean of 10 or 20 readings. Altogether there were made 2470 readings. There exist considerable individual variations in the rate of doing work in the various oysters. Table I shows the frequency distribution of the activities of the oysters; the experi-

mental data are grouped in 14 classes, each at 3 degree intervals, the figures in the body of the table representing the frequencies. It is clear from an examination of the table that the individual fluctuations increase with the temperature and apparently reach their maximum between 24.0° and 26.9°C. where the rate of doing work of different oysters varies from 0.1 to 27.9 ergs per second. In spite of the great individual variations, the results obtained with 15 different oysters

TABLE I.

Frequency Distribution of the Rate of Doing Work at a Given Temperature Interval.

The figures in the body of table indicate the number of determinations. The maxima are printed in heavy type.

	Rate of doing work (ergs/sec.)													
t°C.	0.0–1.9	2.0–3.9	4.0–5.9	6.0–7.9	8.0–9.9	10.0–11.9	12.0–13.9	14.0–15.9	16.0–17.9	18.0–19.9	20.0–21.9	22.0–23.9	24.0–25.9	26.0–27.9
5.0– 5.9	2*													
6.0– 8.9	10													
9.0–11.9	15	3	2	1										
12.0–14.9	3	6	4	2	2	1								
15.0–17.9	4	5	6	2	3	2	2			1				
18.0–20.9	2	3	7	3	5	0	2	3	1		1	1		
21.0–23.9	2	2	2	2	2	3	3	2	2					
24.0–26.9	2	3	2		1		3	3	2	2		1		1
27.0–29.9	1			1			1		2	1				
30.0–32.9		1			1		1			2	1			
33.0–35.9	1	1						2	1					
36.0–38.9				1										
39.0–41.9	2													
42.0–44.9	3*													

* All equal zero.

are consistent in the respect that all the oysters show an increase in the rate of doing work with the increase in temperature, and *vice versa.* Below 40°C. the process is reversible; temperatures above 40°C. produce irreversible changes in the gill epithelium. Oysters kept for 20 minutes at 40° and brought back to 20° failed to recover and produced only slow and irregular currents. Exposure to low temperatures (from 3° to 5°C.) apparently has no injurious effect, the gill epithelium resuming its activity as soon as the temperature is raised.

The relation between the temperature and the rate of doing work is shown in Fig. 3. The curve represents the average results of all the experiments. The data were grouped in 14 classes, each having 3 degree intervals and the true mean of each class was plotted against the mid-value of the class interval. The figures on the curve indicate the number of determinations for each class interval. The results of 4 individual series obtained with 4 different oysters are plotted in

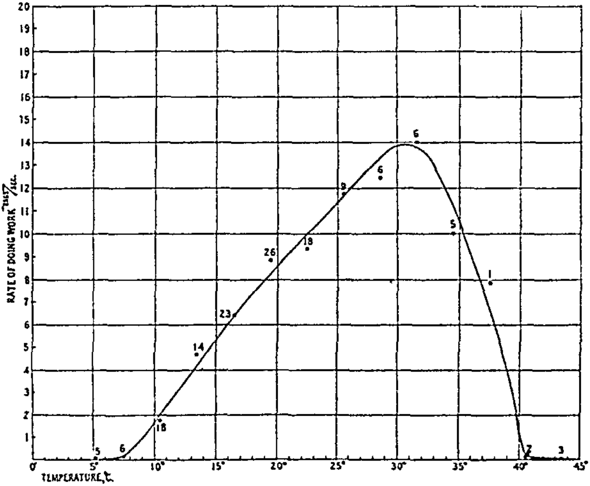

FIG. 3. Effect of temperature on the rate of doing work. Mean of all the experiments. Figures at the plotted points indicate the number of determinations.

Fig. 4. The data of 11 other experiments are not shown because they represent a repetition of one of the curves in Fig. 4.

Examination of the curves shows that the maximum activity of the oyster gill takes place between 25° and 30°C. This is in accord with Gray's data on the effect of temperature on the ciliary motion of a *Mytilus* gill.

Below 7° the efficiency of the ciliary motion of the oyster gill is very

low and comes to zero at 5° while in the *Mytilus* the cilia are still active at this temperature.

The determination of the critical temperature at which the ciliary motion ceases entirely presents certain difficulties. The ciliary activity on the surface of the gill does not stop abruptly when a temperature limit is reached, but slows down gradually, some of the cilia coming to a standstill while the others continue beating. The fact

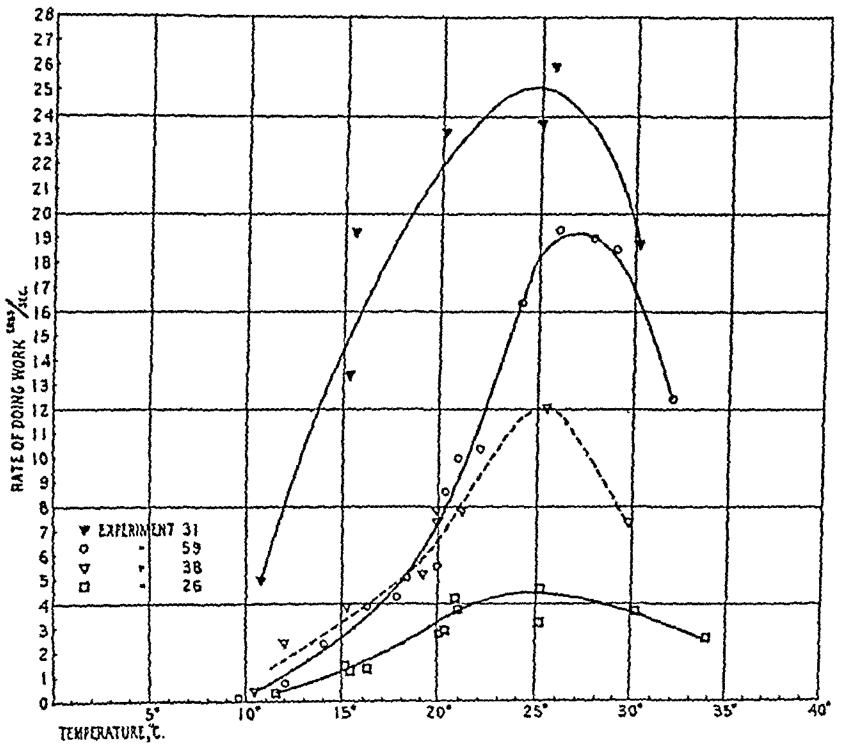

FIG. 4. Effect of temperature on the rate of doing work. Four individual series.

that the current is not produced does not necessarily mean that the cilia are at a standstill; they may beat at such a slow rate or so irregularly that no pressure is maintained in the epibranchial chambers. It frequently happens that the frontal cells are still in motion while all the lateral célls are at a standstill. It has been noticed that in a small fragment of the gill kept under a cover-glass and examined with a high power lens, the ciliary activity usually ceases entirely when the temperature drops to 5°C. (Galtsoff, 1926). Different results, however,

may be obtained when a large piece of the gill is kept in a considerable volume of water and examined with a water immersion lens. In the experiments performed in September, 1926, and February, 1927, at Woods Hole, large pieces of the gills were kept in Stender dishes placed in water jackets filled with a freezing mixture. Under these conditions a complete cessation of the ciliary motion took place at a temperature around $-2°C.$, *i.e.* near the freezing point of Woods Hole water. The action of the cilia at this low temperature is so irregular and slow that apparently it has no significance for the functioning of the gill. The discrepancy between the two sets of experiments should be attributed to the different conditions of the tissues and to the lack of blood in small fragments. No ciliary activity has been observed at temperatures below $-2°C.$; but freezing and exposure to a temperature of $-3.6°$ for 6 hours failed to bring irreversible changes in the ciliary apparatus, and the cilia began to beat as soon as the ice was melted.

In a study of the effect of temperature on various vital phenomena, the question whether the relation between the frequency of the reaction and temperature can be described by the Arrhenius equation undoubtedly is of great interest. The usual procedure is to plot the logarithm of frequencies against the reciprocal of absolute temperature and to determine whether the given series of observations fits a straight line. The individual series of present observations, plotted according to the method adopted by Crozier (1924–25, 1925–26), failed to be rectilinear (Fig. 5), consequently no significant value of the temperature characteristic, μ, can be calculated. Analyzing Gray's data on the effect of temperature on the relative speed of the ciliary motion of *Mytilus* gills, Crozier (1924–25) has shown that they can be described by the Arrhenius formula and he has computed the thermal increment of the reaction. The question naturally arises as to the reason for this discrepancy. Gray's method consisted in determining the time required to move a circular platinum plate over a distance of 1 cm. on the ciliated surface. One of the differences between Gray's experiment and the present investigation lies in the type of the cilia involved. The transport of particles along the surface of the gill is accomplished by the activity of the frontal cilia, while for the current running through the gills the lateral cilia are responsible. It seems improbable, however, that the reactions controlling the activity of two different types

of ciliated cells should be fundamentally different. Undoubtedly all the cilia, in producing certain mechanical work, expend the energy derived from one or several chemical reactions. The work performed by the cilia in moving a plate at a uniform speed is a function of the frequency and the amplitude of the beats. Gray finds that between 0° and 33° the amplitude remains normal while the frequency of the beats increases. Leaving the questions of changes in the resistance

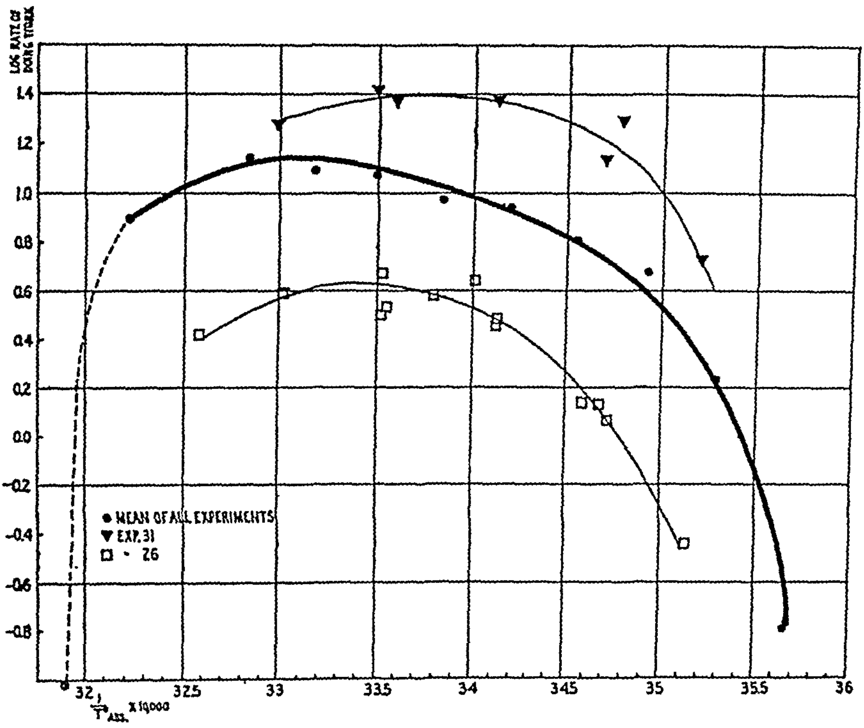

FIG. 5. Logarithms of the rate of doing work plotted against the reciprocal of absolute temperature.

caused by secretion of mucus, the variations in the viscosity of mucus at various temperatures, and the possible changes in the ratio between progressive and regressive strokes (at present we have no method to measure it), the conclusion can be reached that the mechanical work produced in transporting an object over the ciliary surface is a function of the frequency of the beats and depends directly on the velocity of the chemical reaction that controls the ciliary activity.

Certain complications arise, however, when we consider the mechanical activity of the gills in producing a current of water. The flow of water from point A to point B is caused by the difference in pressure between the two points, and the velocity of the current is a function of a pressure drop along the distance AB. The head pressure inside the gill cavity is maintained by the activity of the lateral cilia. As in the case of the transport of particles by frontal cilia, the work produced by the lateral cilia is a function of the frequency of their beats, and depends, also, on the amplitude and the ratio between the progressive and regressive strokes. But the efficiency of the gill in producing a steady current depends not only on these factors but also on the coordination of the ciliary motion along the entire surface of the gill. Under the conditions of the experiments the oysters always have shown certain fluctuations in the rate of flow, which could not be attributed directly to the changes in the surrounding medium. Excluding the cases of mechanical stimulation, the range of fluctuations observed during the experiments varied with the temperature. It has been noticed that the fluctuations are much larger at low or high temperatures than they are within the range from 15° to 25°C. A clear idea of the effect of temperature on the constancy of the current can be gained from an examination of the coefficients of variations $V = \frac{100\sigma}{M}$, where σ = standard deviation and M = arithmetic mean. V has been determined for every set of readings, and all the data were grouped in 11 classes, each having 3 degree intervals. The mean of each class has been plotted at the mid-value of the class interval (Fig. 6). The curve shows very clearly that the absolute variability increases below 15° and above 25°. The fluctuations between 15° and 25° are small, ranging from 4.4 per cent to 5.9 per cent. It is very probable that the observed fluctuations in the rate of flow of water are due chiefly to the temporary disturbances in the coordination of the ciliary activity.

The conditions of the experiments preclude the possibility of a microscopical examination of the behavior of the cilia of a given oyster, but observations made on small fragments of the gill show that at a temperature below 15° the rhythm becomes less regular.

At 10° the characteristic metachronical wave is interrupted frequently because the cilia in some of the filaments begin to beat simultaneously instead of beating in succession, as they normally do.

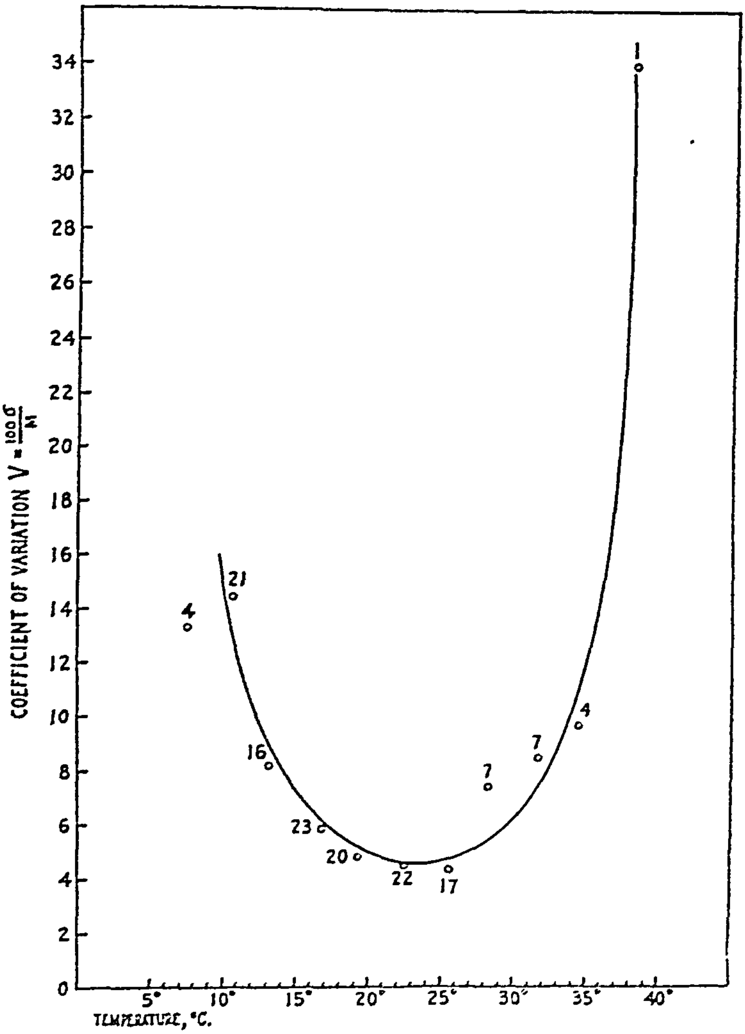

FIG. 6. Relation between the temperature and the absolute variability of the mechanical activity of the gills. Figures at the plotted points indicate the number of determinations.

The result is that in certain blocks of the filament all the lateral cilia beat at once at the same phase, while in the other portion of the filament the metachronical rhythm is maintained. The disturbances in

coordination of the beats, brought about by the changes in temperature, complicate the effect of the latter on the frequency of the beats and supply an explanation of why the Arrhenius equation, which has been found applicable in the case of the relative speed of *Mytilus* cilia, fails to describe the effect of temperature produced on the rate of doing work of the oyster gills. The case is comparable to that of the locomotor activity in the tent caterpillar (Crozier and Stier, 1926), where the relationship between the speed of creeping and the temperature fails to follow the equation of Arrhenius, while the frequency of abdominal peristaltic locomotor waves is controlled by the temperature in accordance with this equation.

The process responsible for the coordination of the ciliary activity has been studied and discussed frequently, but its nature still remains undiscovered. The majority of the workers in this field hold the view that the ciliary motion is not under the control of the nervous system. Several observations to the contrary have been made, however. Merton (1923 and 1924) found that the ciliary epithelium covering the lips in *Physa* and *Helix* is under nervous control and can be set into activity by the stimulation of the proper nerve. Grave and Schmitt (1925), studying the structure of the gill epithelium of fresh water mussels, came to the conclusion that the fibrillar structures in the ciliated cells serve for coordination and regulation of ciliary movement. According to them, the cells may be perfectly autonomous and continue to beat in the absence of neural connections and at the same time there may be "supplementary nervous connections whereby the automatic bent of the cilia is regulated in conformity with the state of the organism as a whole." The authors fail, however, to supply evidence of the existence of the connection between the nervous system and the fibrillar apparatus of the ciliated cells, and the question of the dual nature of the ciliated epithelium requires further investigation. The question whether the coordination of the ciliary motion is due to the neuroid transmission or to the conduction of stimuli through a complex fibrillar mechanism connected to a nervous system has no direct bearing on our problem. The transmission of a stimulus by the epithelium is a well established fact (Kraft, 1890; Wyman, 1924–25) and undoubtedly is of importance for the coordination of the ciliary motion in the gills. The fact that a small piece of

epithelium cut away from the animal body continues to beat with a metachronical rhythm precludes the idea that the rhythmical motion is controlled from some definite point of the tissue. It is very probable that the timing mechanism is situated throughout the whole epithelial layer and, as the present observations show, is affected by exposure to temperatures below 15° and above 25°C.

CONCLUSIONS.

1. The method is described whereby the rate of flow produced by the gills of the oyster can be measured accurately.

2. The rate of doing work in maintaining a constant current along the glass tube can be expressed by the formula $W = 2\pi l \mu S^2$, where W = ergs/sec., l = length of the tube, μ = viscosity in poises, and S = speed at the axis of the tube.

3. The relationship between the rate of doing work and the temperature cannot be described by the equation of Arrhenius.

4. The optimum temperature for the mechanical activity of the gills lies between 25° and 30°C. Below 5° no current is produced, though the cilia are beating. Ciliary motion stops entirely at the freezing temperature of sea water.

5. The factors responsible for the production of current are discussed. The study of the relations between the variability of the rate of flow and the temperature shows that between 15° and 25°C. the absolute variability remains constant and increases considerably above 25° and below 15°. The rôle of the coordination in the production of current is discussed, and the conclusion is reached that coordination is affected by the changes in temperature.

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ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume is appearing simultaneously with Volumes IX and X. Number 1 of this volume will contain a biography of Dr. Loeb. It is to appear after Number 6, and the page numbers will be roman numerals. The publication of this volume began September 18, 1925.

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ON THE RATE OF REACTION BETWEEN ENZYME AND SUBSTRATE.

By JOSEPH BERKSON AND LOUIS B. FLEXNER.

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(Accepted for publication, October 29, 1927.)

"The function of enzymes, as catalysts, being to change the rate of reaction, it follows that the study of their action . . . . , consists essentially in the investigation of the velocity of reaction."

W. M. Bayliss

The investigation reported in this paper is the outgrowth of some experiments which dealt with the antitryptic titre of the blood serum, in certain pathological conditions. In these researches we utilized the method developed by Northrop (1) of gauging enzyme action by viscosity changes of the substrate. We soon reached the conviction that no precise interpretation of our results, or any results derived by similar methods, was possible, without some prior knowledge of the quantitative laws that connected the viscosity changes observed with the enzyme action that brought them about. Accordingly, we inaugurated a series of experiments which looked to the ultimate possibility of stating, in mathematical language, the functional relationship obtaining among all the important variables concerned in the method.

Our first objective was a quantitative description of the rate of change of viscosity as the reaction proceeded from beginning to completion. This we believe we have satisfactorily attained, and our results constitute the content of this report.

Since the viscosity, we suppose, is governed by the size of the gelatin molecule, changes in its value correspond to the hydrolysis of the protein by the enzyme, and the question is equivalent, therefore, to one concerned with the rate of hydrolysis.

Such questions have attracted the attention of investigators for many years.[1] Moore (2) states that O'Sullivan and Tompson were the first observers who studied the velocity of the action of an enzyme, quantitatively, throughout the course of the reaction. Their work (3) was published in 1890, when, employing invertase and cane sugar, they found that the action was monomolecular, following the mass action law, and giving a logarithmic curve. Henri, however, who later worked on the same subject, obtained contradictory results, and found that the percentage rate of change, which is constant in a monomolecular reaction, increased with the progress of hydrolysis. Tammann (4), in a series of researches, investigated not only the action of invertase on cane sugar, but also of emulsin on different glucosides. He found that the velocity was retarded in increasing amount as the reaction proceeded. Duclaux (5) found that with the concentration of the enzyme and sugar fixed, the amount hydrolyzed up to the point at which 20 per cent had been inverted, was simply proportional to the time, so that the curve representing the progress of the reaction up to this point was a straight line and not a logarithmic function. At a later stage, the curve began to obey the logarithmic law. Henri showed afterward that even in this position, Duclaux's results do not give a logarithmic curve, the percentage rate of change all the time increasing with the progress of the reaction.

Henri (6), from an extensive series of experiments on the inversion of cane sugar by invertase, formulated the following equation that predicted the time t, required for a change x, from an original concentration a:

$$K(1 + \epsilon) = \frac{1}{t}\left[\log \frac{a}{a - x} + \log\left(1 + \epsilon \frac{x}{a}\right)\right] \tag{1}$$

ϵ = a factor that refers to the active fraction of the enzyme.
K = a factor that refers to the rate of reaction.
Another formula he developed, supposed to be more generally applicable, is:

$$K = \frac{a}{t}\left[(m - n)\frac{x}{a} + n \log\left(\frac{a}{a - x}\right)\right] + \frac{1}{t}\log\left(\frac{a}{a - x}\right) \tag{2}$$

These equations will be discussed, together with similar ones, in a later part of our paper.

Barendrecht (7), working with invertase and lactose, assuming a certain distri-

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[1] We are indebted to Van Slyke and Cullen (9) for an excellent review of the work done on this subject.

bution of enzyme energy between substrate and products, formulated the following equation:

$$t = \frac{an}{m} \log \left(\frac{a}{a - x}\right) + \left(\frac{1 - n}{m}\right) x \tag{3}$$

Mlle. Filoche (8) found that the action of maltase follows the equation:

$$t = \frac{1}{K}\left(2x + a \log \frac{a}{a - x}\right) \tag{4}$$

Brown and Glendinning have noted in the action of diastase on starch a time curve, which, according to Van Slyke and Cullen (9), is given by the equation:

$$t = \frac{1}{c} \log \left(\frac{a}{a - x}\right) + \frac{x}{a} \tag{5}$$

Abderhalden and Michaelis (10) have plotted the course of the cleavage of *d*-alanyl-*d*-alanine by yeast juice from the results of Abderhalden and Kolliker, and give the following equation as descriptive of the course of reaction:

$$t = \frac{1}{K}\left(\log \frac{a}{a - x} + \frac{\epsilon}{K} x\right) \tag{6}$$

Finally, Van Slyke and Cullen (9), in a carefully argued and exhaustive examination of this question, decided in favor of the equation:

$$t = \frac{1}{\epsilon}\left(\frac{I}{c} \log \frac{a}{a - x} + \frac{x}{a}\right) \tag{7}$$

We may now present our own formulation.

The method of experiment employed follows that of Northrop (1). Gelatin was used as a substrate, and a solution of commercial pancreatin added in an Ostwald viscosimeter maintained at a temperature of 34.5°C. Changes in viscosity were then followed, by noting at successive intervals the time required for the mixture to flow, under the action of gravity, between two points. We call the duration of the fall v, and the time at which the observation is made t. The goal of the inquiry is, then, to discover the mathematical relationship of v and t.

In agreement with previous observers, we found, when low concen-

trations of enzyme were used, that the changes in reaction rate, as reflected in the changes of v, could be represented adequately as an exponential function of t. With stronger solution of enzyme, this was not so; the percentage rate of change departed the more from constancy, the longer the reaction proceeded.

It was experience such as this by previous workers that was the starting point for the development of several of the formulas presented above. The interpretation they made was that the reaction contained different phases, each dominated by a different principle, and equations were constructed on the plan of representing the total action algebraically as the sum of two such elementary processes.

We adopted a different hypothesis as a guiding idea. We assumed that the process was governed throughout by one uniform principle, expressible in a single differential equation containing no summation element. The appearance at times of linearity, and, at others, of an exponential character, we took to signify that the real equation tended to degenerate to these forms under particular circumstances. If the changes described by the equation were minimal, the differences which would distinguish one form from another would become small, and might be obscured by random experimental variation. Our search, then, was for such a mathematical function as would tend to equilibrium with the progress of the reaction, and which would, further, approximate exponentiality and linearity as the process became slower. Several general forms having these characters were exhaustively investigated. We finally decided in favor of the following:

$$V_t - d = \frac{K_1}{1 - K_2 e^{-rt}} \tag{8}$$

V_t = duration of flow of the mixture at time t.
t = the time at which the observation v is made.
r = a parameter reflecting the "intrinsic" rate and depending on the concentration.
K_1 = a parameter equal to the distance between two asymptotes.
K_2 = a parameter determined by d, K_1, and the initial value of v, v_0.
d = a parameter which represents a shifting of the x axis d distance in the y direction.

Diagrammatically the equation is represented in Fig.1.

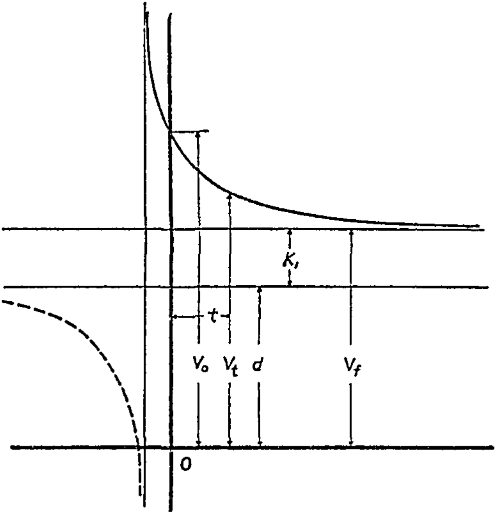

FIG. 1. $v_f = v$ after the proteolysis is complete.

If the logarithm of (8) be derived, we obtain

$$\log\left(\frac{v_t - d - K_1}{v_t - d}\right) = \log K_2 - r \log e \cdot t \tag{9}$$

From this we see that $\log\left(\frac{v_t - d - K_1}{v_t - d}\right)$ plotted against t should give a straight line, the slope of which is $r \log e$. We utilize this fact below, for the evaluation of r.

In order to test the applicability of the equation to our observations, it is first necessary to evaluate the constants, K_1, K_2, and d, in terms of our data. If d were known, this could be accomplished from the following consideration: K_1 = the upper asymptote minus d. By allowing the gelatin to digest until no further change can be noted, the asymptotic value of $v = v_f$ is observed directly, and by subtracting

d, K_1 is obtained. K_2 can then be derived from K_1, d, and the initial reading v_0. The possibility of testing the equation, therefore, turns upon the determination of the quantity d.

By reference to the place that d has in equation (8), it is seen that it is a constant, which, when it is subtracted from each of the observed v's, leaves a residuum, that changes with t. It suggests itself at once that d is the time required for the flow of some constituent of the gelatin mixture inert to the enzyme, and therefore remaining constant through the course of the reaction. We concluded, on the basis of certain of our experimental results, that this was none other than the water itself. Further investigation corroborated this. With d thus established the equation becomes:

$$v_t - v_w = \frac{v_f - v_w}{1 - \frac{v_0 - v_f}{v_0 - v_w} e^{-rt}} \tag{10}$$

in which:

t = time at which the observation is made.
v_t = the duration of flow at any time t.
v_w = v for water.
v_f = the final v after proteolysis is complete.
v_0 = the initial observation of v at $t = 0$.
r = the "intrinsic" exponential rate of increase, obtained from the slope of the line $\log \frac{v - v_f}{v - v_w}$ vs. t
e = the constant 2.71 . . . .

We now apply the formula to two series of our observations, comparing values of v observed, with those calculated from the equation. (Experiments I and II.)

Experiment I.

0.5 cc., 0.15% commercial pancreatin sol. + 10 cc. 3% gelatin—by viscosity method.

Temperature 34.5°C.
v_0 = 59.5 sec. observed.
v_w = 29.5 " "
v_f = 35.5 " "
r = 0.006518 per min., calculated from slope of

$$\log \frac{v - v_f}{v - v_w} \text{ vs. } t$$

From (10) the equation becomes for this case:

$$v_t = \frac{6}{1 - 0.800\, e^{-0.006518\, t}} + 29.5 \tag{11}$$

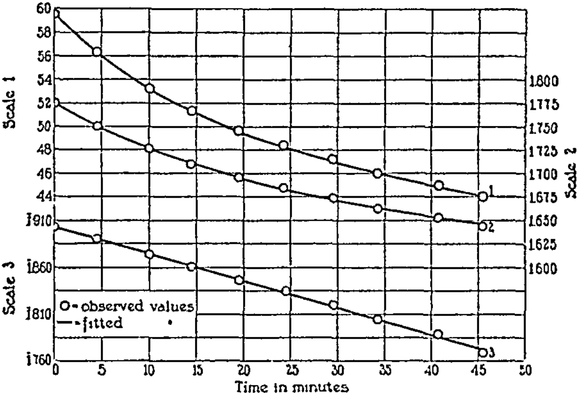

FIG. 2. Experiment I. 0.5 cc. 0.15% pancreatin + 10 cc. 3% gelatin.

1. Arithmetic graph, v vs. t — $v_f = 35.5$ sec. } observed.
2. Exponential " $\log v$ vs. t — $v_\infty = 29.5$ " } observed.
3. Autocatalytic " $\log \frac{v - K_1}{v}$ vs. t — $K_1 = v_f - v_\infty = 6.0$; $r = 0.006518$ from slope.

t	v_t Observed	v_t Calculated from (11)
min.	*sec.*	*sec.*
0	59.5	59.5
4½	56.3	56.4
10*	53.2	53.4
14½	51.3	51.5
19½	49.6	49.8
24½	48.4	48.4
29½	47.2	47.1
34½	46.0	46.1
40¾	45.0	45.0
46½	44.4	44.1

Standard deviation calculated from observed = ±0.15 sec.

Coefficient of variation = ±0.3%

Experiment II.

0.5 cc., 0.25% commercial pancreatin sol. + 10 cc. 3% gelatin – by viscos method.

Temperature 34.5°C.

v_0 = 51.6 sec. observed.

v_w = 30.5 " "

v_f = 36.2 " "

r = 0.0092876 per min., calculated from slope of

$$\frac{v_t - v_f}{v_f - v_w} \text{ vs. } t$$

The equation becomes for this case:

$$v_t = \frac{5.7}{1 - 0.72986\, e^{-0.0092876\, t}} + 30.5$$

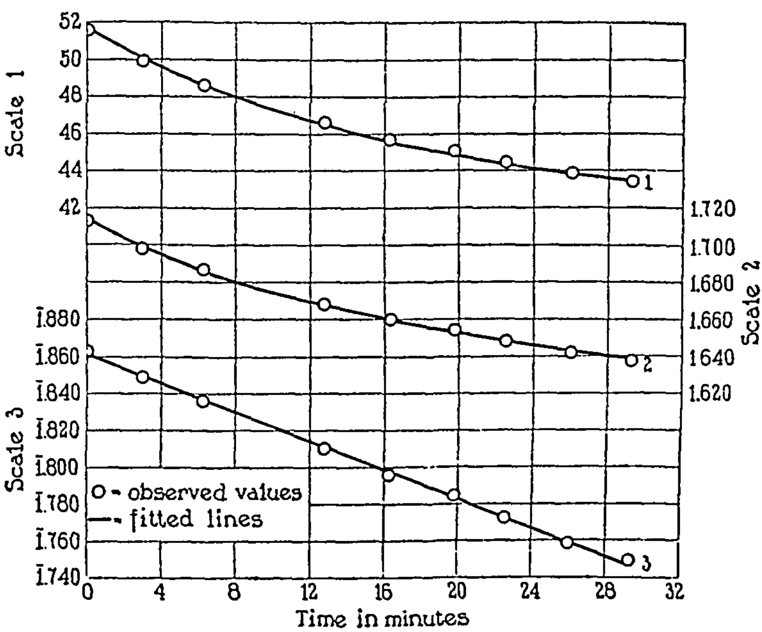

FIG. 3. Experiment II. 0.5 cc. 0.25% pancreatin + 10 cc. 3% gelatin.

1. Arithmetic graph, v vs. t — $v_f = 36.2$ } observed.
2. Exponential " $\log v$ vs. t — $v_w = 30.5$ } observed.
3. Autocatalytic " $\log \frac{v - K_1}{}$ vs. t — $K_1 = v_f - v_w = 5.7$; $r = 0.0092876$ from slope.

	v_t	v_t
	Observed	Calculated from (12)
min.	*sec.*	*sec.*
0	51.6	51.6
3°	49.9	50.1
6¼	48.6	48.8
12¾	46.6	46.8
16¼	45.7	45.6
19¾	45.1	45.0
22½	44.5	44.5
26°	43.9	43.9
29¼	43.5	43.4

Standard deviation calculated from observed = ± 0.13 sec.
Coefficient of variation = ± 0.3%

Such tests of the equation have now been made by us upon many series of observations and the precision of prediction shown in the above experiments is typical.

The examples here referred to are ones in which the rate of hydrolysis is rapid. When the concentration of the enzyme used is low, and the velocity of reaction correspondingly slow, the equation describing the process approaches the exponential and it becomes impossible to differentiate it experimentally from one which is a simple monomolecular. This may be shown theoretically as follows:

Using differential expressions, the value of v determined from the exponential would be:

$$v_t = r \int_0 v \, dt \tag{13}$$

where $v_t = v$ at the time t, r = rate constant. Determined from (8) it would be:

$$v_t = \frac{r}{K_1} \int_0^t v(v - K_1) \, dt \tag{14}$$

The difference between the values thus determined would, therefore, be:

$$\text{Diff.} = r \left[\int v dt + \frac{1}{K_1} \int v(v - K_1) dt \right]_0^t \tag{15}$$

It is seen that the difference diminishes in proportion as r decreases, *i.e.* when the reaction is a slow one. We may expect, therefore, that

as we use an enzyme solution of greater dilution the reaction will appear logarithmic. This proves to be the case experimentally.

In illustration, if we examine a series of observations made with a mixture of gelatin and dilute pancreatin it will be found that they can be well represented by a logarithmic curve. But it is equally possible to fit a curve of form (8) to them and we present such a fit for the same experiment. The explanation is simply that in this instance r is so small, that the difference between the two functions is of the order of the experimental error, and so is inappreciable.

Experiment III.

0.5 cc., 0.025% pancreatin plus 10 cc. 3% gelatin — by viscosity method.
Temperature 34.5°C.
$r = 0.0032$ per min., from exponential graph, $\log v$ vs. t.
$v_0 = 66.4$ sec. observed.
Exponential equation is, therefore:

$$v_t = v_0^{-rt} = 66.4\, e^{-0.0032t} \tag{16}$$

$v_\infty = 27.1$ sec. observed.
$K_1 = 12.8$, $K_2 = 0.674$ — calculated.[2]
$r = 0.0032$ per min. from exponential function.
Autocatalytic equation is therefore from (8):

$$v_t = \frac{12.8}{1 - 0.674\, e^{-0.0032t}} + 27.1 \tag{17}$$

t	v_t From exponential function (16)	v_t From autocatalytic function (17)	v_t Observed
min.	*sec.*	*sec.*	*sec.*
0	66.4	66.4	66.4
4½	65.5	65.3	65.4
8½	64.6	64.3	64.4
13½	63.6	63.3	63.3
18¼	62.6	62.3	62.6
22¾	61.7	61.4	61.7
27°	60.9	60.6	60.7
31½	60.1	59.9	60.0
36°	59.1	59.2	59.6
40¼	58.3	58.5	58.6

[2] V_f could not in this case be directly observed because of the deterioration of the pancreatin itself during the long period required for complete hydrolysis.

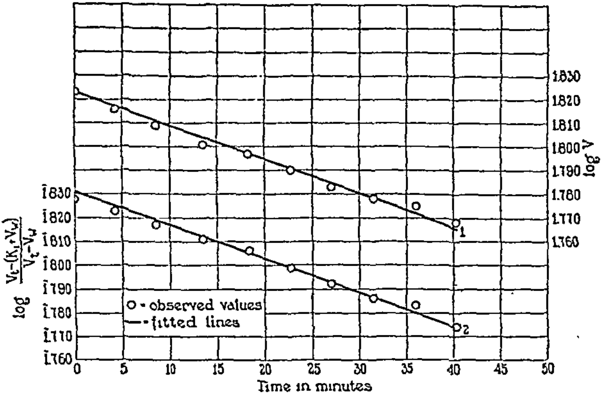

FIG. 4. Experiment III. 5 cc. 0.025% pancreatin + 10 cc. gelatin. Viscosity method.

1. Exponential function, log v vs. t. $r = 0.0032$ per min. from slope.
2. Autocatalytic function, log $\frac{v_t - (K_1 + v_w)}{v_t - v_w}$ vs. t

$v_w = 27.1$ sec., observed.
$K_1 = 12.8$, $K_2 = 0.674$ } calculated.
$r = 0.0032$ per min. from slope.

On the basis of the results reviewed and many others of similar character, we believe that an equation of form (10) is a true quantitative description of the viscosity changes in a mixture of gelatin and pancreatin. Further, we are convinced by these results, and by evidence about to be considered, that it refers to the proteolytic process and, indeed, is necessarily consequent to the laws that govern the velocity of enzyme reactions in general. The grounds for this conviction will now be considered.

If equation (8) be differentiated, the following is obtained:

$$\frac{dv}{dt} = \frac{r}{K_1} (v) (v - K_1) \tag{18}$$

This states that the rate of change of viscosity (as measured by change in duration of flow) is proportional at any instant, to the value at that instant of v, and v minus a constant K_1. Now it can easily be shown that this is identical in general form with the differential equation obtained by Ostwald (11) to describe a process which he called "autocatalytic." Characteristic of such a reaction, is the fact that a change in the amount of substrate is accompanied by a change (in his case an increase) in the amount of enzymatic activity.

We are able to prove that an equation of form (18) results from assuming a reaction between gelatin and pancreatin of such nature, that the change of given amount of gelatin is accompanied by the inactivation of a fixed quantity of enzyme.

$$n_1 \text{ gelatin}_1 + n_2 \underset{\text{active}}{\text{enzyme}} \rightleftarrows n_3 \text{ gelatin}_2 \cdot \underset{\text{inactive}}{\text{enzyme}} \tag{19}$$

Let E_0 = initial concentration of the enzyme.
e_a = the concentration of active enzyme at any moment.
e_i = " " " inactive " " " "
G_0 = the initial concentration of gelatin.
g_1 = the concentration of unchanged gelatin.
g_2 = " " " proteolyzed "
n_1, n_2, n_3 = stoichiometric factors.

We make the following assumptions:

(1) That the change in the direction $g_1 \leftarrow g_2$ is negligible so that the reaction $g_1 \rightarrow g_2$ is practically irreversible.

(2) That the velocity is governed by the law of mass action.

(3) That v is proportional at any moment to the concentration of unchanged gelatin.

We have, then:

$$\frac{dg}{dt} = -k_1 (g_1)(e_a) = -k_1 (g_1)(E_0 - e_i) = -k_1 (g_1)\left[E_0 - \frac{n_2}{n_3}(G_0 - g_1)\right]$$

$$= -\frac{n_2}{n_3} k_1 (G_1)\left(G_1 - \frac{n_2 G_0 - n_3 E_0}{n_2}\right) \tag{20}$$

$\dfrac{n_2}{n_3} k_1$ and $\dfrac{n_2 G_0 - n_3 E_0}{n_2}$ are constants and if we suppose v proportional to the unchanged substrate we have:

$$\frac{dv}{dt} = -C_1 (v)(v - C_2) \tag{21}$$

Equation (21) is identical with (18) and is the differential equation of (8) and (10). We have been able to show, therefore, that equation (8), which is found to agree precisely with our observations, follows from the assumption of a simple bimolecular reaction between pancreatin and substrate. How widely applicable are these conclusions?

Although we have experimented with gelatin and pancreatin alone, and utilized only the viscosity method of gauging the progress of the reaction, we are of the opinion that enzyme changes in general follow fundamentally the same process, and that this would be supported by other methods of estimating their action, *e.g.* surface tension, electric conductivity, polarimetric method,[3] etc. The results of many experimenters employing a wide variety of substrates and methods have been examined. We have not, in general, been able to make a direct test of the applicability to them of equation (8) for want of certain measurements necessary to the determination of the required constants. But no instance has come to our notice in which the form of changes observed could not be explained on the assumption of an autocatalytic reaction defined by (8).[4] Happily, in a few cases, the circumstances of the experiment, and the manner of presentation of the data, permit a direct test, and for these the results follow.

The data tested comprise:

(1) Three series of observations by Van Slyke and Cullen (9) in which urea and urease were used and the amount of NH_3 produced determined by titration.

(2) One series of observations by Brown and Glendinning (12) in which starch and diastase were employed, and the changes followed by determining the cupric reducing power of the products.

(3) One series of Abderhalden and Michaelis (10) in which the splitting effect of yeast juice on polypeptid was measured by the polarimetric method.

In each of these, we found, the results did not agree with an autocatalytic equation such as fitted our own observations. This, however, was a limited example, and applied to the case when the enzyme

[3] Experiments to test this are planned.

[4] We mean, in this statement, that the constants be permitted plus or minus signs.

was decreasing. If we assume instead, that the enzymatic activity is increasing the corresponding equation becomes:

$$x' = \frac{K_1}{1 + K_2\, e^{rt}} \tag{22}$$

x' = amount of substrate remaining at time t.
t = time at which the observation is made.

If we begin the experiment at the moment of greatest possible velocity it may be shown that K_1 equals twice the original value of x' and K_2 equals 1, and we have

$$x' = \frac{2 \cdot x_0}{1 + e^{rt}} \tag{23}$$

Experiment IV.

Van Slyke and Cullen (9).
0.02 mol. sol. urea plus 0.03% enzyme—by titration of NH_3 produced.
$K_1 = 2 \times 40 = 80$
$r = 0.0033492$ from slope of $\log\left(\frac{K_1 - x'}{x'}\right)$ vs. t
From (23) the equation for this case becomes:

$$x' = \frac{80}{1 + e^{0.0033492\, t}} \tag{24}$$

x' = units of NH_3 combined as urea $\left(1 \text{ cc. } \frac{N}{10}\ NH_3 \text{ per } 10 \text{ cc. sol.} = 1 \text{ unit}\right)$.
t = time of observation, min.

Observed by Van Slyke and Cullen		Calculated from equation (24)
t	x'	x'
min.	*units* NH_3	*units* NH_3
60	35.80	36.01
120	31.60	32.08
186	27.90	27.93
240	25.16	24.74
300	21.92	21.44
360	18.90	18.44
420	16.30	15.74

Standard deviation observed from calculated = ±0.43 min.
Coefficient of variation = ±1.7%

Experiment V.

Van Slyke and Cullen (9).
0.02 mol. sol. urea plus 0.1% enzyme—by titration of NH_3 produced.
$K_1 = 80$ as in Experiment IV.
$r = 0.011585$ from slope of $\log\left(\frac{K_1 - x'}{x'}\right)$ vs. t

Equation (23) becomes:

$$x' = \frac{80}{1 + e^{0.011585\,t}} \tag{25}$$

Observed by Van Slyke and Cullen		Calculated from equation (25)
t	x'	x'
min.	*units* NH_3	*units* NH_3
15	36.20	36.52
30	32.76	33.12
45	29.48	29.99
60	26.72	26.63
75	23.70	23.63
90	21.10	20.86
105	18.50	18.29
120	16.60	15.95
135	14.64	13.85
150	12.46	11.97
165	10.42	10.30
180	8.70	8.84
195	7.20	7.57
225	5.46	5.72

Standard deviation observed from calculated = ±0.38 units.
Coefficient of variation = ±2.0%

Experiment VI.

Van Slyke and Cullen (9).
0.02 mol. sol. urea plus 0.3% enzyme—by titration of NH_3 produced.
$K_1 = 80$ as in Experiment IV.
$r = 0.036406$ from slope of $\log\left(\frac{K_1 - x'}{x'}\right)$ vs. t

From (23) the equation for this case becomes:

$$x' = \frac{80}{1 + e^{0.036406\,t}} \tag{26}$$

Observed by Van Slyke and Cullen		Calculated from equation (26)
t	x'	x'
min.	units NH_3	units NH_3
20	25.92	26.04
30	19.78	20.09
40	14.94	15.12
51	10.80	10.81
60	8.02	8.09
75	4.88	4.90
90	3.10	2.91
105	2.00	1.71
120	1.00	1.00
135	0.46	0.58

Standard deviation observed from calculated = ± 0.168 units.
Coefficient of variation = ± 1.9%

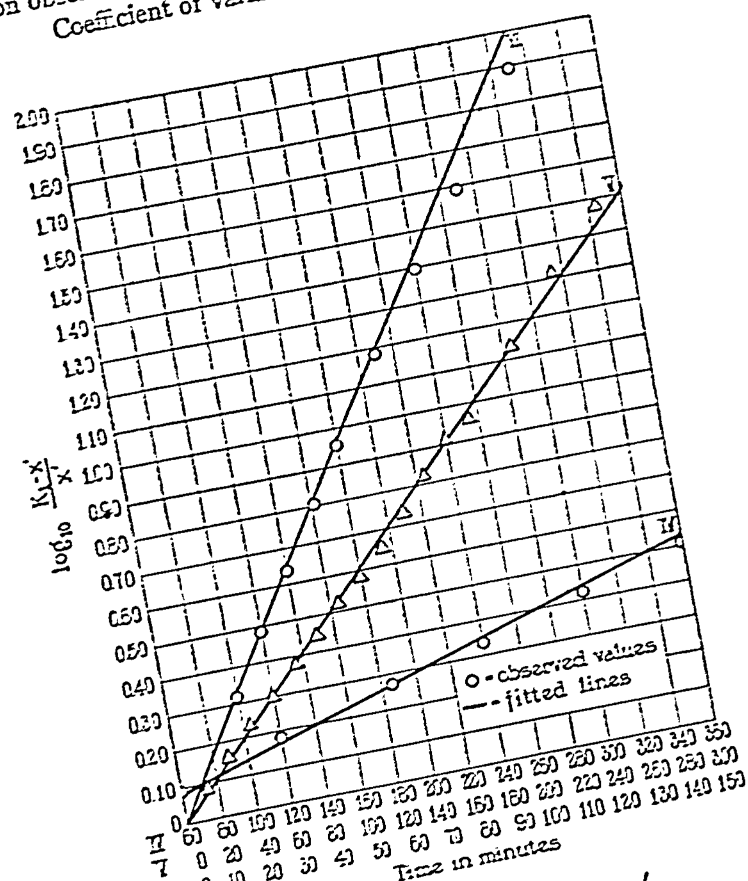

FIG. 5. Autocatalytic graphs. $\log_{10} \dfrac{K_1 - x'}{x'}$ vs. t.

Experiment IV. 0.03% enzyme. r, from slope = 0.0033492.
" V. 0.1% " r, " " = 0.011585.
" VI. 0.3% " r, " " = 0.036406.
From data of Van Slyke and Cullen. Urea and urease, by titration.

Experiment VII.

Brown and Glendinning (12).
3% starch sol. plus 0.25% malt extract—by cupric reducing power of products.
$K_1 = 2 \times 1 = 2$
$r = 0.023337$ from $\log\left(\frac{K_1 - x'}{x'}\right)$ vs. t
Equation (23) for this case becomes:

$$x' = \frac{2}{1 + e^{0.023337\,t}} \tag{27}$$

x' = units of unchanged starch.
t = time at which observation was made, min.

Observed by Brown and Glendinning		Calculated from equation (27)
t	x'	x'
min.	*units*	*units*
10	0.892	0.884
20	0.775	0.771
30	0.665	0.664
40	0.565	0.565
50	0.465	0.475
60	0.385	0.396
70	0.320	0.327
80	0.262	0.268
90	0.220	0.218
100	0.185	0.177
110	0.150	0.142
120	0.120	0.115
130	0.097	0.092
140	0.078	0.073
150	0.060	0.053
160	0.050	0.046

Standard deviation calculated from observed = ±0.0064 units.
Coefficient of variation = ±2.0%

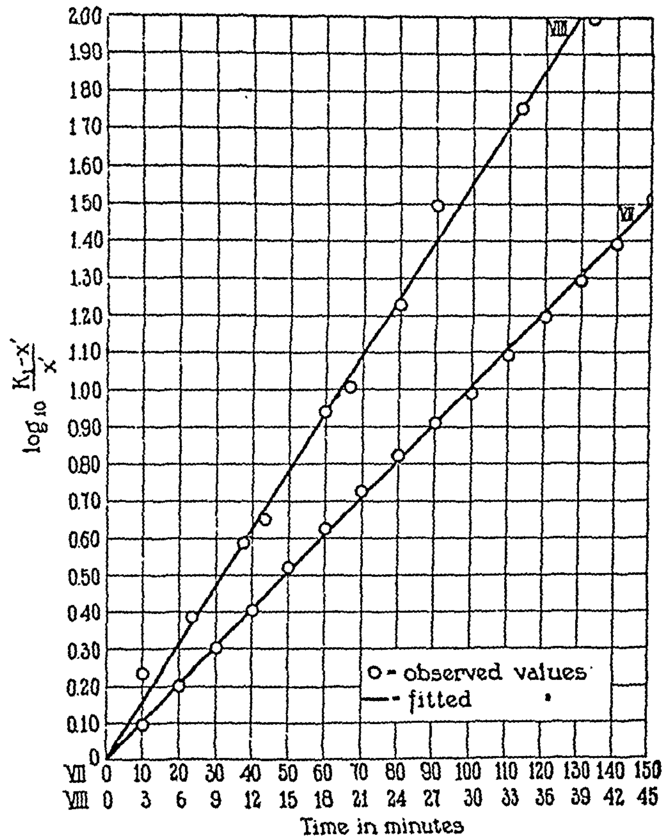

FIG. 6. Autocatalytic graphs. $\log_{10} \dfrac{K_1 - x'}{x'}$ vs. t.

Experiment VII. From data of Brown and Glendinning. Starch and diastase. r, from slope = 0.023337.

Experiment VIII. From data of Abderhalden and Michaelis. Dipetid and enzyme. r, from slope = 0.11827.

Experiment VIII.

Abderhalden and Michaelis (10).
1.45 units of dipeptid plus 6 cc. enzyme—by polarimetric method.
$K_1 - 2 \times 1.45 = 2.90$
$r = 0.11827$ from slope of $\log \left(\dfrac{K_1 - x'}{x'}\right)$ vs. t

Equation (23) for this case becomes:

$$x' = \frac{2.9}{1 + e^{0.11827\,t}} \tag{28}$$

x' = the amount of unchanged dipeptid in units of optical rotation.
t = time at which observation was made, min.

Observed by Abderhalden and Michaelis		Calculated from equation (28)
t	x'	x'
min.	*units*	*units*
0	1.45	1.45
3	1.20	1.06
7	0.88	0.84
11	0.62	0.61
13	0.51	0.53
18	0.31	0.30
20	0.25	0.26
24	0.16	0.16
27	0.11	0.09
34	0.05	0.05
40	0.03	0.03

Standard deviation observed from calculated = ±0.017
Coefficient of variation = ±3.7%

In all these experiments, as was the case with our own, the deviations of observed from calculated values from the autocatalytic equation is of the order of the experimental error involved in the method used.

Comparison with Other Formulas and Discussion of the Autocatalytic Equation.

A review of the more important equations that have been formulated to represent the reaction between enzyme and substrate has been made above. They may be analyzed to be of three kinds. First, those equating the time for a change x to a logarithmic function of x. Second, those equating the time to the sum of a linear and logarithmic function of x. Third, the first formula of Henri, which stands by itself and equates the time to the sum of two logarithmic functions of x.

The first is the ordinary exponential form, and is the one corresponding to a monomolecular reaction. It was abandoned by all experimenters who worked over considerable ranges of concentration of enzyme, on the grounds that it did not accord with observed results. We may discard it, as an unsatisfactory form for general application, with the note that in our experience with low concentrations of enzyme, as with other experimenters, working in the realm of slowly proceeding changes, this form was found to apply satisfactorily. An illustration of this was given in our Experiment III.

The second group, that giving the time as the sum of a logarithmic and an arithmetic function of x, is the largest and warrants more careful analysis. We may generalize the experience of those workers who were persuaded to formulate this kind of equation, somewhat as follows:

They found that in certain reactions in their entirety, and in portions of others, the changes proceeded at a sensibly constant rate, giving a linear relationship between x and t. In other situations the relationship was curvilinear, the rate of changes varying rapidly. In the case where a reaction showed both features, neither linear nor exponential was applicable throughout. An exponential equation fitted to the rapidly changing portion did not suit the linear part and any rule that described the straighter region, broke down when applied to that with greater curvature. Then they noted that a function consisting of the sum of two parts, a linear and logarithmic, would suit all the observations more or less, the constants being so chosen that the linear part of the function applied to the constant part of the reaction and the logarithmic became important when the reaction rate varied.

We think this gives a fair picture of the methodology involved in all the formulas under discussion. They differ among themselves according to the mode of choice and interpretation of the constants.

Now, while these formulas will, if the proper constants are inserted, give good approximate fits, this of itself is no vindication of their form. We have compared, where feasible, predictions made by means of them, and by our own, and in each case tested ours gives significantly better values. But the important distinction rests not in the greater precision of prediction so much as in the simplicity of form of the autocatalytic and in the economy and character of the constants used.

In our equation (23) which is comparable with (7) of Van Slyke and Cullen, there are two as against four constants. Further, if we consider the more general form (10) used in our own experiments, each of the constants involved has definite physical meaning and with the exception of r[5] is determined by an experiment quite independent of the series to which it is later applied. These considerations lead to the further fact that the differential equation is of a simple form, and requires a minimum of assumptions for its derivation. In contradistinction, the differential equation derived from the sum of an arithmetic and logarithmic function, requires a complexity of assumptions for its establishment and has resulted in the construction of involved theories respecting the nature of enzyme action that have no independent corroboration.

The remaining equation to be considered, (1) that of Henri, may be shown to reduce to an autocatalytic form when certain of the constants are combined. It was, in fact, derived from Ostwald's differential equation for just this reaction. The form elaborated by Henri is to be criticized for the introduction of constants which are not necessary and which destroy the virtue of simplicity of the original.

The autocatalytic form utilized by us, we believe, gives an adequate quantitative description of the velocity changes in the reaction between enzyme and substrate. The generality of its application is reconciled with the exponential and linear changes that other experimenters and we have noted in some instances, by the fact that it tends to approximate these forms when r assumes small values. It can be deduced from the simple assumption of a bimolecular reaction, obeying the mass law. In every case tried the prediction made on the basis of it, deviates from observation by no greater amounts than involved in the experimental error. This expresses the fact that it is as close an approximation to the truth as is permitted to us by the available methods of measurement. It is what characterizes a law; and if the experimental results presented above should be verified for enzymes in general, it would establish the equation as the law governing such reactions.

[5] Correlated with concentration of enzyme—see note following this paper

CONCLUSIONS.

1. An equation of the form:

$$v_t - v_w = \frac{v_f - v_w}{1 - \frac{v_0 - v_f}{v_0 - v_w} e^{-rt}} \tag{10}$$

in which v_t is the time of flow of the mixture, v_w the time of flow for water, v_f the time of flow of the mixture when proteolysis is complete, v_0 the time of flow at the beginning of the experiment, t the time of observation, and r a constant, has been found to describe accurately the course of change of viscosity in a mixture of gelatin and pancreatin.

2. An equation of the same general form has been found to apply similarly to the reaction between other enzymes and other substrates.

3. The equation may be derived theoretically from assuming a bimolecular reaction between enzyme and substrate obeying the mass action law.

We wish to thank Dr. Wilburt C. Davison, of the Department of Pediatrics, under whom this investigation was conducted, for the interest and encouragement he has given our work, and for the generous permission granted at the cost of much personal inconvenience, to use his laboratory and instruments.

To Dr. Lowell J. Reed, of the Department of Biometry and Vital Statistics, is acknowledged great indebtedness for advice sought by us throughout the investigation, respecting the mathematical problems that arose. His suggestions have been incorporated in the paper at many points, though we alone remain responsible for any errors in the final statement.

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APPENDIX.

A NOTE ON THE RELATIONSHIP OF *r* TO THE ENZYME CONCENTRATION.

The authors are pursuing researches to establish the functional relationships of the rate parameter *r* of the autocatalytic equation to other variables. They have not yet completed either the analysis of the questions involved or the experimental work necessary. But they believe they have sufficient evidence to warrant the tentative conclusion that as respects the relationship of *r* to the concentration of the enzyme used, the correlation is linear.

In substantiation, we present, in graphic form, the results of four series of experiments, as follows:

Series A.—Concentration against *r*; five of our experiments with gelatin and trypsin, using viscosity, in which the reaction was slow, and the *r* calculated from the exponential function.

Series B.—Ditto A, the reaction rapid and *r* calculated from the autocatalytic function.

Series C.—Three experiments of Van Slyke and Cullen, using urea and urease, in which the data were taken from their article[6] and *r* calculated in each case from the autocatalytic function.

Series D.—Four experiments of Abderhalden and Michaelis,[7] using dipeptid and enzyme in which the data were taken from their article, and *r* calculated from the autocatalytic function.

[6] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.
[7] Abderhalden, E., and Michaelis, L., *Z. physiol. Chem.*, 1907, lii, 326.

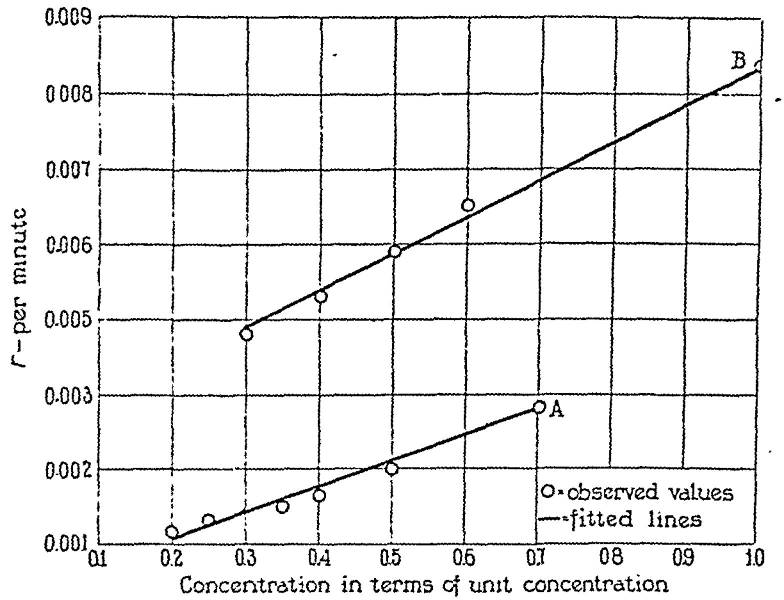

FIG. 7. Concentration of pancreatin vs. r. Gelatin and pancreatin. Viscosity method.

Series A. r from exponential function about 0.4% pancreatin = unity.

Series B. r " autocatalytic " " 0.5% " = "

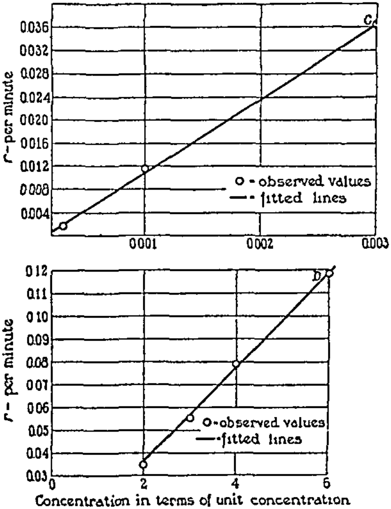

FIG. 8. Concentration vs. *r*.

Series C. Data of Van Slyke and Cullen. Urea plus urease. 20% extract soy bean = unit concentration urease. *r* = from autocatalytic function.

Series D. Data of Abderhalden and Michaelis. Dipetid plus enzyme. *r* = from autocatalytic function.

THE RATE OF REDUCTION OF METHYLENE BLUE BY BACILLUS COLI.

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(From the Biochemical Laboratory, Cambridge, England.)

(Accepted for publication, November 17, 1927.)

It has recently been shown (1) that the velocity (v) of the hydrolysis of starch by the amylase of germinated barley varies with the concentration (c) of the starch according to the equation

$$v = a + b \log c$$

where a and b are constants. A similar relationship has been described (2) for the reduction of methylene blue by "resting" *B. coli* using succinic acid as substrate. As these are the only instances described of this relationship it seemed of interest to compare them more closely, and for this purpose it was necessary to elaborate the data for the latter case.

Methods.

The organism used was "resting" *B. coli* prepared as described by Quastel and Wooldridge (3); *i.e.*, it was grown on tryptic broth, centrifuged and washed with 0.95 per cent sodium chloride several times, and a current of air was passed through the suspension for several hours. It was kept at 0° until diluted and used.

The buffers were phosphate (Clark and Lubs) and the pH value was checked electrometrically. Succinic acid was brought to the required pH by the addition of sodium hydroxide before being used.

The various reagents were mixed in several tubes of the kind described by Thunberg (4), and finally the suspension of bacilli was added. After putting in the stoppers all the tubes were connected to the same pump and evacuation was begun as quickly as possible. After thorough evacuation the tubes were filled with nitrogen and the evacuation repeated. They were then placed in the water bath at 45° as nearly at the same time as possible. Before this plan of simultaneous evacuation was adopted, great difficulty was experienced in obtaining results of any regularity, since the degree of evacuation varied, *e.g.* with the water pressure in the pump, and even minute traces of oxygen affect the reduction times very markedly. It may be noted that the amount of methylene blue used in a

tube was only 2×10^{-5} gm. An experiment was done to show the effect of incomplete evacuation and will be given later. In order to minimize the danger of leakage etc., the suspension of the organism was adjusted so that the reduction was complete within 2 hours.

As shown by Quastel and Whetham (5), the rate of decolorisation of the methylene blue is linear until approximately 95 per cent of the methylene blue is reduced, and accordingly a standard was used containing 0.05 or 0.10 cc. of the methylene blue solution with the same amount of bacterial emulsion and saline made up to volume. This of course was not evacuated. Consequently the time for 90 or 95 per cent reduction was used as a measure of the rate.

Even with these precautions there remain numerous sources of error, and control experiments show that differences in similar tubes are often 5 per cent, but seldom exceed 10 per cent.

TABLE I.

Succinate concentration	Reduction time	
	Found	Calculated
M/120	9′ 30″	8′ 33″
M/240	10′	11′ 5″
M/360	12′ 55″	12′ 49″
M/480	14′ 10″	14′ 20″
M/600	16′ 20″	15′ 58″
M/720	17′ 20″	17′ 32″
M/840	20′ 20″	19′

Succinic Acid.

As Quastel and Whetham discovered this relationship with succinic acid, I first repeated their experiments. A typical result is given in Table I.

The calculations in Table I are made from the equation

$$v = 25.74 - 7.01 \log c.$$

Since the agreement is quite satisfactory, and since in none of the curves obtained is there any definite trend of the divergences between found and calculated values, it may be concluded that equations of this type accurately describe the relations found over this range of concentrations.

An interesting part of the amylase curve is with low concentrations of starch where zero velocity is obtained. Unfortunately I was unable to compare the two curves in this region, because in the case of *B. coli* the danger of slow leakages, etc., render prolonged experiments unsatisfactory; and although I have obtained with low concentrations of succinate results that appeared to indicate that the time for reduction was becoming infinitely long and the velocity therefore zero, I have no confidence that I have completely excluded the possibility of serious errors.

A note may be inserted here on the effect of incomplete evacuation. Four identical sets, each of three tubes, were prepared, in which the final succinate concentrations were M/100, M/300 and M/500. Each succeeding set was evacuated more thoroughly than the preceding. The results are given in Fig. 1 where the velocity ($= 100/t$ where t is the time in minutes for 95 per cent reduction) is

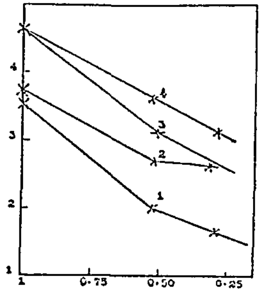


FIG. 1. The effect of incomplete evacuation. Curve 1 belongs to the least evacuated and so in order to No. 4 the most thoroughly evacuated.

plotted against the logarithm of the succinate concentration. It is obvious that with more thorough evacuation there is a decrease in reduction time and an approach to regularity. The slope of the curve also changes a good deal, and considerable caution must be used in drawing deductions from alterations in the slope of the curve.

Glucose.

To test the limits of validity of this relation another substrate of different chemical character was now substituted for succinic acid. Results with glucose are given in Fig. 2. The smooth curve is drawn from the equation

$$v = 22.8 - 4.83 \log c.$$

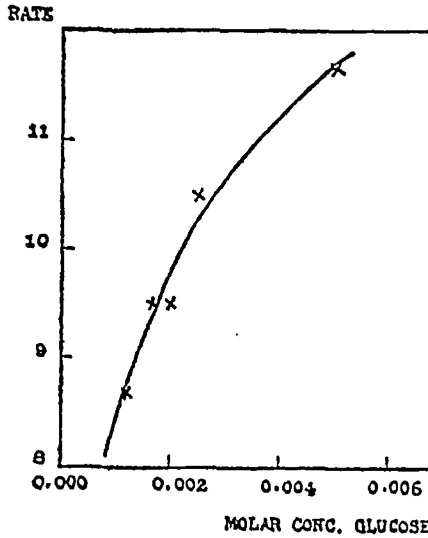


FIG. 2. The reduction velocity of glucose at moderate concentrations. x = experimental points. The smooth curve is drawn from the equation.

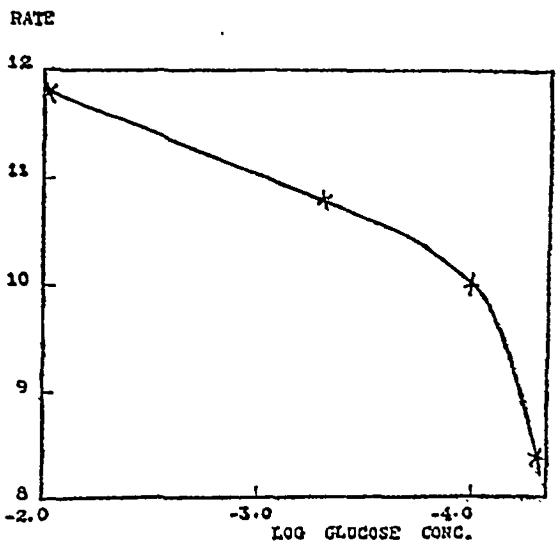
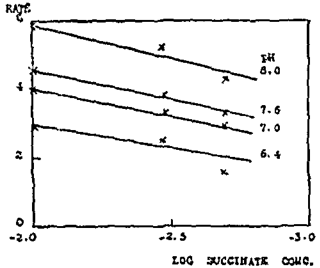


FIG. 3. The reduction velocity of glucose at low concentrations, showing how the velocity falls off more rapidly than is predicted by the equation. Values fitting the equation form a straight line as on the left of the diagram.

it is obvious that this correctly describes the relations over this ;e of concentrations. However with concentrations less than this e is a rapid decrease in velocity, until one reaches concentrations hich the amount of glucose is less than sufficient for the reduction ıe methylene blue present. This is illustrated in Fig. 3. The rithm of glucose concentration is used so that deviations from the rithmic relation will show as deviations from a straight line. The e of this decrease in velocity with low concentrations may possibly difference in the rate of transference of the different hydrogens of ose. The number transferred, however, is (as might be expected) pendent of the concentration of methylene blue.



ɜ. 4. The effect of pH on the relation between substrate concentration ːeduction velocity.

may be noted incidentally that here we have complete reduction cc. of 1:5,000 methylene blue in the presence of 5 cc. of M/40,000 ose; this means that each molecule of glucose gives up almost ːtly 5 atoms of hydrogen to the methylene blue. Since it is highly robable that the number of hydrogens donated is odd, we assume one molecule of glucose donates six hydrogens, one being transːd to the oxygen still remaining. This would require 0.0007 cc. xygen. Quastel and Whetham have demonstrated that four or e atoms were donated and considered that the number is probably

Effect of Hydrogen Ion Concentration.

Differences between the amylase system and the *B. coli* system are clearly shown when one varies the pH. Results from one experiment are shown in Fig. 4. Four series of three tubes were prepared, the pH of the different series being 6.4, 7.0, 7.6 and 8.0, and the succinate concentrations of the different tubes of each series being M/100, M/300 and M/500. In plotting, the logarithms of these concentrations were used, and the result is four parallel lines. In other words, the effect, within these limits, of making the solution more alkaline is to increase the rate of reduction; and this increase, being proportionately the

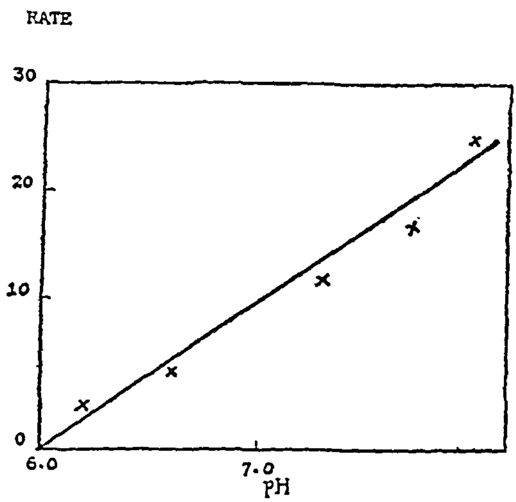


FIG. 5. The effect of pH on reduction velocity.

same, is independent of the concentration of the substrate. With barley amylase the effect on the rate is relatively more marked at lower concentrations, and the results when plotted in this way show lines which are not parallel.

Quastel and Whetham (5) found that when the reduction time was plotted against pH, a curve resembling a rectangular hyperbola was obtained. I have used a much narrower range of pH than they did. With glucose as donator there was little or no difference in rate between pH 6.3 and 7.7; on the acid side of this the rate falls off. The results with succinic acid are shown in Fig. 5. Here velocity is directly proportionate to the pH value.

In attempting to analyse this relationship, we may consider the succinate, the methylene blue and the organism. Alteration of pH over this range has no effect on the succinate except to increase slightly its degree of ionisation, a change too slight to produce these results.

Clark, Cohen and Gibbs (6) have shown that the E_h for 50 per cent reduction of methylene blue alters in a linear way with the pH over this range, the former going from positive to negative with increasing alkalinity. Now, if (Cannan, Cohen and Clark, (7)) methylene blue and similar dyes adjust practically instantaneously to induced levels of oxidation-reduction potential, and the lag in reduction in experiments such as these is due to the fact that the cell system gradually develops greater negative potential, then we should expect, others things being equal, that the reduction will occur first in those solutions in which the E_h for 50 per cent reduction of the methylene blue is least negative. In other words the least alkaline solutions will become reduced first. The exact opposite, however, is the case, and we must conclude that the effect of the alteration in pH is on the organism and its enzymes, and that the effect on the methylene blue is completely masked. The only alternative to this is to suppose that under these conditions the behaviour of cell suspensions differs from that predicted by W. M. Clark and his co-workers. In order to test this point I used their series of indicators. (I am indebted to Dr. M. Dixon for these dyes. I understand that they were sent to Dr. J. Needham by Dr. W. M. Clark.) The concentrations of dye solutions were equivalent to the concentration of the methylene blue used in previous experiments. The substrate used was succinate. Table II gives the results of three experiments. The concentration of the *B. coli* suspension and succinate was different in each of them.

These experiments show (*a*) that Nos. 1, 2 and 3 are not reduced; (*b*) that No. 7 is the fastest, being markedly faster than No. 8 (this dye contains no halogen nor sulfonic acid group); (*c*) that methylene blue (No. 3*a*) in Experiment *a* occupies a place close to No. 8, in Experiment *b* close to No. 7, while in Experiment *c* it is slower than No. 6. Thus not only does it not occupy its proper place in the series but its position is variable. Apparently when the rate of

reduction of the whole series is faster, the rate for methylene blue is relatively faster still.

The anomalous behaviour of Nos. 3*a* and 7 thus seems to indicate that the reduction of the dye is not entirely independent of the reducing system or the substance acting as hydrogen donator, as Clark appears to think. On this point see Dixon (8). The variable position of methylene blue may perhaps be accounted for by attributing to it a slight toxic action. It should be noted that this would be more pronounced in experiments of longer duration and it is in these that the reduction of methylene blue is relatively slowest. This, however, will not explain its very rapid reduction in some cases.

TABLE II.

Indicator	Rate of reduction (= 100/*t*)		
	Experiment *a*	Experiment *b*	Experiment *c*
1. Indigotin disulphonate	0	0	—
2. Indigotin trisulphonate	0	0	—
3. Indigotin tetrasulphonate	0	0	—
3a. Methylene blue	5.3	30.7	Less than 0.5
4. 1-naphthol-2-sulphonate-2-6-dichloroindophenol	0.5	1.0	—
5. 1-naphthol-2-sulphonate indophenol	1.4	3.0	—
6. o-cresol, 2-6-dichloroindophenol	4.0	9.2	1.0
7. o-cresol indophenol	14.3	50.0	4.4
8. 2-6-dibromophenol indophenol	5.0	11.1	1.2

Effect of Temperature.

This was studied with succinate as substrate. A typical result is as follows.

Succinate concentration	Q_{10} (37° – 47°)
M/20	2.3
M/120	1.8

The slight falling off of the Q_{10} with lower concentration (where the duration of the experiment was necessarily longer) suggests a slight injurious effect of the higher temperature on the organism. There is here again a striking difference from the amylase-starch system.

DISCUSSION.

Summing up the comparison of this system with that of barley amylase and starch, we find that, within limits, the same relation between substrate concentration and velocity holds for both of them. Beyond this differences appear; and the effects of alteration in pH and temperature on this relation are quite different in the two cases.

There still remains the question of the significance of the equation relating velocity to substrate concentration. If we assume that the mechanism of the reaction is essentially a combination of the substrate with its enzyme, and that this combination decomposes to give the products of the reaction, we may regard the velocity as proportional to the amount of this combination existing in the mixture. This combination may be chemical (*cf.*, *e.g.*, invertase (9), liver amylase (10)), or physical, *i.e.* of the nature of adsorption. If chemical, it will be governed by the mass law, and we should expect the relation between velocity and substrate concentration to be of the type described by Michaelis and Menten (9). Since it very obviously does not conform to this, we may consider the possibility of adsorption. Several equations have been proposed to describe the relation of the amount of a substance adsorbed to its concentration in solution, such as those of Freundlich, Arrhenius, Langmuir; but as Garner (11) points out all these equations have only a limited range of validity. Some of them, *e.g.* that of Freundlich, as pointed out by Arrhenius (12) have no theoretical basis. Arrhenius in the same paper deduced an equation from a characteristic property of adsorption, *viz.*, that the amount of substance adsorbed reaches a maximum value. If we suppose that this limit is reached asymptotically we may deduce an equation giving the same general sort of curve. Let us suppose, then, that on increasing the amount of substance in solution the amount adsorbed (x) shows gradually smaller increases as the concentration in solution (c) rises. This may be written as a differential equation:

$$\frac{dx}{dc} = \frac{p}{c}$$

p being a constant. On integrating this we obtain

$$x = p \cdot \log c + q$$

where q is the constant of integration. This, of course, is identical with the equation I found empirically. The significance of the equation therefore appears to be that there is a gradual approach to a saturated condition. The constant q indicates that there is no adsorption ($x = 0$) until the concentration reaches a definite value ($c = e^{-q/p}$). In other words there is a sudden break in the curve at this point. In this connection it is of interest to note that Garner (11) finds breaks in the adsorption isotherms of certain alcohols.

The equation therefore probably means that adsorption plays an essential part in the reaction.

SUMMARY.

This paper deals with the relation between substrate concentration and velocity in the case of the reduction of methylene blue and of the other oxidation-reduction indicators of Clark by *B. coli* in the presence of succinic acid and glucose. This system is compared with starch and barley amylase. Reasons are given for considering the mechanism as an adsorption phenomenon.

It is a pleasure to acknowledge my indebtedness to Prof. Sir F. G. Hopkins and to Dr. J. H. Quastel.

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THE OXYGEN CONSUMPTION OF LUMINOUS BACTERIA.

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(Accepted for publication, January 18, 1928.)

In a previous paper (1925) I have published some figures on the total efficiency of luminous bacteria, regarded as machines for light production, which give the ratio of the light emitted to the total energy input, expressed in the same energy units (calories per second). The energy input was calculated by a method of indirect calorimetry, from the oxygen consumption of the bacteria. The oxygen consumed would liberate a definite amount of heat when used to burn the food of the bacteria, a mixture of 40 per cent peptone and 60 per cent glycerol.

Since luminescence of the bacteria is dependent on dissolved oxygen, the oxygen consumption was measured by finding the time necessary for an emulsion of the bacteria in sea water to use up the dissolved oxygen to the point where the luminescence just begins to fade. From the solubility of oxygen in sea water one can then calculate the oxygen consumption, provided the bacteria use equal amounts of oxygen in equal times. Experiment shows that the dimming point represents utilization of at least 99.5 per cent of the dissolved oxygen, a change from the dissolved oxygen in equilibrium with air (21 per cent) to that in equilibrium with < 0.5 per cent oxygen, and that the luminescence intensity of the bacteria is unaffected by different oxygen tensions until the tension falls to < 0.5 per cent oxygen.[1] The oxygen consumption is probably also independent of the oxygen tension until approximately this same value is reached. The time for dimming for an undisturbed tube of bacterial emulsion is proportional to the number of bacteria present, and also approximately proportional to the oxygen tension with which the bacterial emulsion is brought into equili-

[1] Unpublished experiments.

brium, provided of course this is above the critical value (0.5 per cent oxygen). The method would therefore appear to be justified, but requires confirmation, which is the subject of this communication.

The actual figures obtained (Harvey, 1925) for oxygen consumption of the bacteria in seven experiments at temperatures ranging from 18° to 23°C. varied from 6.65 to 17.67 $\times 10^{-15}$ mg. O_2 per bacterium per second, with an average of 11.84 $\times 10^{-15}$ mg. for an average temperature of 21.5°C. These values are 10 per cent too low, since it was believed, at the time the experiments were made, that 2 per cent oxygen was the point at which dimming occurred.

In order to check this method I have recently made determinations of oxygen consumption in the Thunberg-Winterstein respirometer as used by Fenn (1927) for experiments on oxygen consumption by nerve.[2] The two chambers were of approximately 22 cc. capacity and the capillary held 0.403 gm. of mercury in a length of 153 mm. Since the oxygen consumption in this apparatus is twice the volume movement of the capillary, each mm. movement of the oil drop in the capillary was equivalent to the consumption of 0.000388 cc. O_2. The chambers contained small vessels for the solution of KOH to absorb CO_2. Two cc. of bacterial emulsion in sea water was placed in one chamber and 2 cc. sea water was placed in the other, both in equilibrium with air. The whole apparatus was immersed in a thermostat of running sea water at 21°C. which changed less than 0.01°C. over a period of 2 hours. The readings of the oil droplet movements plotted against time are given in Fig. 1, together with three determinations of the time for another sample of the same emulsion of luminous bacteria to dim, when standing undisturbed at the same temperature.

From Fig. 1 it will be seen that the rate of oxygen consumption is practically independant of rate of shaking and that 2 cc. of bacterial emulsion used 0.0272 cc. O_2 in 1950 seconds or 0.0000139 cc. O_2 per second. One cc. of emulsion would use 0.00000695 cc. per second.

Over the same time interval, at 21°C., it took 12.5 minutes or 750 seconds (time indicated by arrows) for the luminescence of a similar bacterial emulsion to dim. At 21°C. each cc. of sea water will dis-

[2] I am greatly indebted to Dr. W. O. Fenn for the micro respirometer used in these experiments and to Mr. C. S. Shoup for growing the luminous bacteria and for assistance with the experiments.

solve 0.0052 cc. O_2 (Fox, 1909), at least 99.5 per cent of which is consumed when dimming occurs. Hence the bacteria in 1 cc. sea water emulsion used 0.0052 ÷ 750 or 0.00000693 cc. O_2 per second, a value in perfect agreement with the micro respirometer. I conclude that the dimming method gives a very good index of the oxygen consumption of the bacteria.

It is interesting to compare the oxygen consumption of luminous bacteria with that of other bacteria. Of the numerous papers on

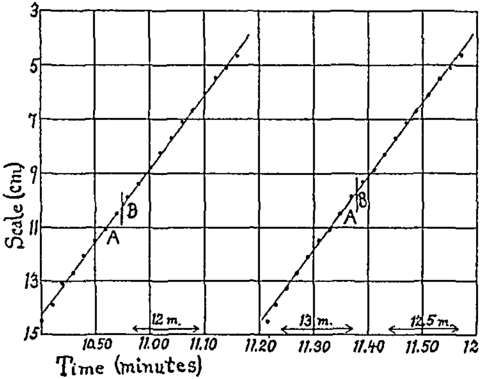

Fig. 1. Readings of index drop scale of micro respirometer in cm. plotted against time in minutes. Below the mark, (*A*), the respirometer was shaken 98 times per minute; above, (*B*), at 120 times per minute. The horizontal arrow lines indicate the times for bacterial emulsions to use dissolved oxygen, as determined by the dimming method.

respiration of bacteria (*cf.* Stephenson and Whetham (1924), Novy and Soule (1925), Brooks (1918–1922), Callow (1924) and Pütter (1924)), only Callow and Pütter give values which can be properly compared, namely, oxygen consumption per weight or volume of bacteria. The luminous bacterium used by me in efficiency studies was a cylinder averaging 2.2μ long by 1.1μ in diameter with rounded ends. Its surface area was 7.6μ^2 and its volume 1.695μ^3. When

TABLE I.

	Organism	Observer	Volume in μ^3	Surface in μ^2	Temperature	Oxygen consumption in mg. per hr.		
						Per individual	Per kg.	Per sq. m.
Aerobic	*Photobacterium phosphorescens*	Harvey, 1925	1.7	7.6	21.5°	4.26×10^{-11}	2.5×10^{4}*	5.6
	Bacillus fluorescens non-				15°	1.8×10^{-10}	3×10^{6}	143
	liquifaciens	Pütter, 1924	0.06	1.26	21°	3.56×10^{-10}	5.9×10^{6}	280
	Bacillus fluorescens liquifaciens	Müller, 1912	0.19	1.90	22°	7.64×10^{-10}	4.01×10^{6}	400
	Various bacilli	Callow, 1924					1 to 5.4×10^{3}†	
Facultative anaerobic	*Bacillus coli*	Müller, 1912	0.47	3.9	22°	1.28×10^{-10}	0.271×10^{6}	33
	Yeast	?	180	150	26°	27.3×10^{-10}	0.0168×10^{6}	20
	Colpidium	Pütter, 1905	153×10^{3}	16.9×10^{3}	17°	0.368×10^{-6}	2250	23

* On the assumption of a density = 1 for *Photobacterium phosphorescens.*

† On the assumption of 85 per cent water in bacteria, since Callow's results are based on dry weight of bacteria.

oxygen consumption per gm. of luminous bacterium is calculated (assuming a density of 1) we obtain an average value of 0.291 cc. O_2 per gm. per minute or 17.46 cc. O_2 per hour. Callow finds 5 to 25 cc., O_2 per gm. dry weight per hour, for several types of aerobes. Assuming 85 per cent water in the bacterium, these figures become 0.75 to 3.75 cc. O_2 per gm. moist bacterium per hour.

Calculating the average oxygen consumption in terms of a single bacterium, per kilo of weight and per sq. m. of surface, we obtain the values given in the accompanying table. We see that the oxygen used per surface area and per kilo is very much less than the values found by Pütter, although greater than those recorded by Callow.

Pütter has pointed out that when oxygen consumption per sq. m. of respiring surface is compared, different groups of organisms whose size varies greatly give similar values. I am inclined to question the validity of this conclusion except as a very general statement. However, much of the data which has accumulated on unicellular forms and single cells would have far greater value had the experimenters taken the trouble to determine the number, surface and volume (or weight) of the cells studied.

The most important point in comparing the oxygen consumption of cells is to have sufficient oxygen available so that the center of the cell will receive diffusing oxygen more rapidly than it can be used. Only if this is true will oxygen consumption be independent of oxygen tension. Too great oxygen tensions, as pure oxygen, should also be avoided, since there is evidence, at least in the case of these luminous bacteria, that oxygen consumption may be slowed under these conditions.[1]

Gerard (1927) and Fenn (1927) have both published the equations, $C_0 = \frac{Ar^2}{4D}$, for calculating the oxygen pressure, C_0, in atmospheres at the surface of a long cylinder respiring at a definite rate which allows all parts to receive adequate oxygen. In this equation, A is the oxygen consumption of the tissue in cc. oxygen per gm. per minute, r is the radius of cylinder and D is the diffusion coefficient of oxygen for the tissue in cc. oxygen diffusing per sq. cm. for a pressure gradient of one atmosphere per cm. The equation becomes $C_0 = \frac{Ar^2}{6D}$ for a sphere

and approximately $C_0 = \frac{Ar^2}{5D}$ for a short cylinder such as the luminous bacterium.[3]

Using Krogh's (1919) diffusion coefficient of oxygen for connective tissue, 1.15×10^{-5}, and an oxygen consumption of 0.29 cc. per gm. per minute, with $r = 0.000055$ cm., C_0 turns out to be 1.53×10^{-5} atmospheres. Remembering that the bacteria are surrounded by sea water and not air, we must allow for the solubility of oxygen in sea water. One cc. sea water will dissolve 0.0255 cc. oxygen at N. T. P. from one atmosphere of pure oxygen at 20°C. Hence $1.53 \times 10^{-5} \div 2.55 \times 10^{-2} = 0.6 \times 10^{-3}$ or 0.0006 atmosphere oxygen in equilibrium with sea water should maintain an adequate respiration of the bacteria. Let us assume that the point of inadequate oxygen supply is the point where luminescence begins to dim.[4] As this value (0.0006 oxygen) is about 4 times smaller than the value (approximately 0.0026) at which we observe the luminescence to begin to dim, I am led to believe that the diffusion coefficient, the only value in the equation not directly measured, is too high. It seems very likely, then, that oxygen penetrates far less readily into a luminous bacterium than into gelatin gel or connective tissue. It is very possible that these cells are surrounded by a chitin-like envelope as some text-books of bacteriology assert. Krogh (1919) found the diffusion of oxygen through chitin to be about 9 times as slow as through connective tissue.

SUMMARY.

Oxygen consumption of luminous bacteria determined by the Thunberg micro respirometer and by the time which elapses before the luminescence of an emulsion of luminous bacteria in sea water begins to dim, when over 99 per cent of the dissolved oxygen has been consumed, agree exactly.

Average values for oxygen consumption at an average temperature

[3] This equation was derived for me by my colleague, Dr. J. W. Alexander. Inasmuch as the assumption is made in the derivation that oxygen consumption is independent of oxygen concentration for *all* oxygen concentrations, it is questionable if this equation can be applied to an object as small as a bacterium.

[4] This question is under investigation at present.

of 21.5°C. are 4.26×10^{-11} mg. O_2 per bacterium; 2.5×10^4 mg. per kilo and 5.6 mg. O_2 per sq. m. of bacterial surface.

The only correct comparison of the oxygen consumption of different organisms or tissues is in terms of oxygen used per unit weight with a sufficient oxygen tension so that oxygen consumption is independent of oxygen tension.

Measurement of the oxygen concentration which just allows full luminescence, compared with a calculation of the oxygen concentration at the surface of a bacterial cell just necessary to allow the observed respiration throughout all parts of the cell, indicates that oxygen must diffuse into the bacterium much more slowly than through gelatin or connective tissue but not as slowly as through chitin.

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PREPARATION OF ELECTROLYTE-FREE GELATIN.

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The method described here is a modification of the one employed by Loeb. His method consisted principally in bringing powdered gelatin, by means of acetic acid, to a pH slightly below the isoelectric point and then washing it several times with distilled water. This process brought about a complete removal of various cations. No special care was taken to remove anions except so far as they were replaced by the acetate ion of the acetic acid. In our modified method, of which a brief description was given in a former publication,[1] the gelatin is treated, in addition to acetic acid, also with dilute alkali, and then washed at pH 4.7 with H_2O, thus bringing about a removal of both cations and anions. The details of the process are as follows.

1000 gm. of Cooper's non-bleached powdered gelatin, the fine dust of which has been removed by screening it through an 80 mesh sieve, are soaked for 1 hour, with constant stirring, in 20 liters of M/128 acetic acid, at a temperature of 5° to 10°C. A glazed 5 gallon earthenware jar immersed in a water bath cooled by means of a coil from a refrigerating machine is employed as a container. The gelatin is washed three times with cold distilled water by allowing the gelatin grains to settle, removing the sypernatant liquid as thoroughly as possible, and then refilling the container. The supernatant liquid is conveniently removed by the use of an inverted Büchner funnel, 14 cm. in diameter, connected by means of a long rubber tubing to a 4 liter suction flask and then to a suction pump. A padding made of towel cloth is spread on the funnel in order to filter out the gelatin. The suspension of gelatin is stirred for about 20 minutes in the washing liquid before renewing it.

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 354.

After the last washing the container is refilled to the mark with cold H_2O and a definite amount of strong NaOH solution of a known strength (80 cc., 5 M) is added to the gelatin so as to bring the total strength to about M/50 alkali. The NaOH solution is added slowly while the gelatin is thoroughly stirred. The gelatin is allowed to remain in the alkaline solution for 1 hour. The supernatant solution is then removed and the gelatin washed three times with cold H_2O in the same manner as after the treatment with acetic acid. But care is taken to keep track of the amount of NaOH removed by the washings by measuring the volume of liquid decanted and also titrating samples of it with HCl. Thus the total amount of NaOH left in the gelatin solution is definitely found.

Acetic acid of a known strength, in amount double that of the *NaOH left* in the gelatin, is then added to the gelatin. This brings the gelatin to pH 4.7. The gelatin mass, which had been greatly swollen in the alkali, shrinks considerably at this stage, and the removal of electrolytes by washing with cold distilled water proceeds rapidly. After four or five washings the specific conductivity of the settled gelatin when melted is from 3 to 5×10^{-5} reciprocal ohms at 35°C. Its concentration is about 17 gm. per 100 cc. solution. The gelatin, after it has been sufficiently washed and the washings removed, is stirred up with about 5 liters of 95 per cent alcohol for about 15 minutes. It is afterwards transferred to a large Büchner funnel, perfused once or twice with fresh alcohol and then with ether. It dries easily in the air when spread out in a thin layer on filter paper. A solution of 2.3 gm. of the dry gelatin in distilled water had a pH of 4.84 and a specific conductivity of 1.5×10^{-5}. The specific conductivity of the water used was 3.4×10^{-6}.

Further washings do not affect the conductivity to any considerable degree. One of the effects of too much washing is the removal of the final traces of diffusible anions which are required to keep the water in which the gelatin is dissolved at pH 4.7, with the result that the gelatin in dilute solutions is negatively charged.[2] If it is desirable to

[2] Dhéré (Dhéré, C., *Kolloid Z.*, 1927, xli, 315) found this to be the fact also in case of purification of gelatin by electrodialysis. He concludes that the isoelectric gelatin of Loeb is not isoelectric at all but a combination of gelatin with ions. Apparently Dhéré fails to appreciate the fact that since at the isoelectric point of

keep the pH at the isoelectric point it is necessary to dilute the gelatin with distilled water of pH 4.7.

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gelatin (pH 4.7) there is an excess of hydrogen ions over the OH ions, there must of necessity be enough diffusible anions to keep the solution electrically neutral. A removal of the last trace of the diffusible ions gives rise to negative protein ions until electrical neutrality is reached. The greater the dilution of the gelatin, the further will the solution be removed from pH 4.7 towards the pH of the distilled water used for dilution.

COMBINATION OF SALTS AND PROTEINS.

III. The Combination of $CuCl_2$, $MgCl_2$, $CaCl_2$, $AlCl_3$, $LaCl_3$, KCl, $AgNO_3$ and Na_2SO_4 with Gelatin.

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The combination of proteins with acids and alkalies has been thoroughly studied by a number of investigators, but the reaction of proteins with salts has received little attention—owing largely to the lack of a convenient method for determining the free ion concentration of those ions for which there is no satisfactory electrode. The problem is of interest since salts have almost as marked an effect on the properties of protein solutions as do acids. It was pointed out in the previous papers[1] of this series that the activity of an ion in a protein solution could be determined by setting up a Donnan equilibrium and measuring the total ion concentration in the pure salt solution outside the membrane, and in the protein solution inside the membrane. If the membrane potential is also measured the ratio of the activity of the ion inside to that outside can be calculated and if the activity coefficients are known then the concentration of the ion inside can be calculated. The difference between this figure and the total concentration found by analysis evidently gives the concentration of combined ion; *i.e.*,

$$M_c = M_t - \frac{M_o \gamma_o}{r \gamma_i}, \tag{1}$$

where M_t is the total concentration of ion found by analysis inside, M_c is the concentration of combined ion, M_o is the total concentration

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 25; 1925–26, ix, 351.

of ion outside and γ_o, γ_i are the activity coefficients of the ion in the outside and inside solutions respectively, and *log* $r = .4343 \dfrac{\text{Membrane Potential}}{RT/nF}$. In the present experiments the total salt concentration differs only slightly between the inside and the outside solution, so that if the effect of the protein on the ionic strength of the solution is neglected[2] then $\gamma_o = \gamma_i$.

Experimental Procedure.

The experiments were carried out as already described except that a more sensitive galvanometer was used. The membrane potentials were measured on four systems and two other systems were analyzed gravimetrically, Cl as AgCl, Cu as CuSCN, Ca as Ca oxalate, Mg as $Mg_2P_2O_7$ and Al as $Al(OH)_3$. Duplicate determinations were made on both the inside and outside solutions of both systems. Experiments in which the analyses differed by more than 0.2 per cent or the potential measurements by more than 0.05 millivolt were not used. The figures given are therefore the averages of 2 analyses of each solution and of four potential measurements. Even under the best conditions, however, the error in the final figure may amount to 10 or 20 per cent, especially in the concentrated salt solutions, since the calculated amount of ion combined depends on a small difference between two large experimental figures.

The isoelectric gelatin was prepared as described in the preceding paper.[4]

Preparation of Deaminized Gelatin.—100 gm. of isoelectric gelatin were dissolved in 1 liter of water and 10 gm. $NaNO_3$ and 15 cc. glacial acetic acid added, then warmed at 80° for 4 hours, at the end of which time all amino nitrogen had been removed.

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[2] As pointed out in the previous paper[1] the experiments give directly the decrease in the activity of the ion in the presence of the protein, but in order to calculate the actual change in concentration of the ion it is necessary to make some assumption as to the effect of the protein on the activity coefficient (*i.e.* on the ionic strength of the solution). In the present calculation it has been assumed that this effect is negligible.

At first sight this appears an improbable assumption since the protein, although present in small concentration, is undoubtedly polyvalent. It may be noted, however, that according to Simms[3] the effective valence of polyvalent ions decreases as the distance between the groups increases, so that gelatin in acid solution has an effective valence of 1.8. It was also shown that the results obtained in this way agree with those obtained for Cl, H^+ and Zn^{++} by means of concentration cells.

[3] Simms, H. S., *J. Gen. Physiol.*, 1927–28, xi, 613.

[4] Northrop, J. H., and Kuntzi, M., *J. Gen. Physiol.*, 1927–28, xi, 477.

TABLE I.

Combination of Copper Chloride and Gelatin.

Concentration of gelatin	Ion determined	P.D.	Concentration of Cu^{++} Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin		
			Inside	Outside	Cu^{++}	Average	Cl^-
per cent		*mv.*					
1.8	Cl	+2.5	10.7	8.8	0.19		0.10
4.5	Cl	8.1	13.1	8.1	.20		.10
			13.2	8.0	.19		
4.5	Cl	5.8	23.8	16.6	.28		.14
4.5	Cl	3.5	54.4	43.95	.44		.18
4.5	Cu	1.8	107.6	94.5	.58		.30
			107.4	91.4	.63	.60	
1.8	Cl	.75	101.3	95.8	.60		.32
1.8	Cu^{++}	.50	185 0	179.0	.72		
			184.0	177.0	.78		
4.5	Cu^{++}	1.1	203.0	187.5	.66	.75 ±.02	.35
			206.2	188.3	.72		
1.8	Cl	.50	199.5	193.0	.78		.28
			199.5	192.0	.84		
4.5	Cu^{++}	.80	355.0	329.0	.98		
			356.0	330.0	.98	.93 ±.03	
1.8	Cu^{++}	.26	342.0	334.0	.83		
1.8	Cl	.15	494.0	484.0	.89		
			497.0	486.0	.92	.90 ±.01	
4.5	Cl	.63	495.2	479.2	.90		.23
			497.5	479.0	.89		.31

The solution was kept at 20° for 26 hours and then dialyzed at 30° in a ro dialyzing apparatus.[5] Final solution: 6 gm. per 100 cc. deaminized ge specific conductivity 7×10^{-5}: pH (30°) 4.1; H^+ combined at pH 1.51 = millimols per gm.

EXPERIMENTAL RESULTS.

Copper Chloride.—Copper is known to form complexes with monia and amino groups so that it seemed reasonable to suppose

TABLE II.

Deaminized Gelatin. 1.7 Per Cent. pH 4.1.

Concentration of gelatin	Ion determined	P.D.	Concentration of Cu^{++} Millimols per 1000 gm. H_2O		Millimols combined per gm. gela		
			Inside	Outside	Cu^{++}	Average	(
per cent		*mv.*					
1.7	Cl	.20	194.1	191.8	0.33		0.
1.7	Cl	.10	293.0	290.5	.31		.
			295.0	292.0	.34		.
1.7	Cl	.12	388.0	383.8	.50	.44 ±.03	.
			385.0	383.8	.30		.
			388.0	384.0	.50		
			385.0	382.0	.45		
1.5	Cl	.07	491.9	486.5	.49		.
6.0	Cl	.34	515.0	500.0	.46		
6.6	Cl	.35	723.0	690.0	.50		
5.5	Cl	.35	713.0	708.0	.51		
5.5	Cl	.28	925.0	909.0	.63		
4.3	Cl	.28	930.0	930.0	.47		

it would be found to combine with proteins. The results of the exp ments with this salt are shown in Table I. The table shows that copper ion is combined to a large extent in dilute solution and t on increasing the copper concentration the copper combined per g of gelatin increases and reaches a maximum at about 0.9 millim per gm., which agrees with the equivalent combining power of gela for hydrogen ion. In other words, the reaction is stoichiomet

[5] Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1927–28, xi, 641.

since 1 mole of copper is equivalent to 1 mole of hydrogen. Apparently then copper combines with the same groups in the protein molecule as does hydrogen. This conclusion can be verified by determining the combining power of deaminized gelatin. It was shown by Hitchcock[6] that hydrogen ions reacted stoichiometrically with the

TABLE III.

HCl. $CuCl_2$. Gelatin.

Combination of Hydrogen and Copper.

1.8 Per Cent Gelatin.

pH	P.D.	Final concentration of Cu^{++} Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin		
		Inside	Outside	Cu^{++}	H^+	$Cu^+ + H^+$
	mv.					
4.7	0.70	104.6	96.6	0.70		0.70
		105.5	96.4	.80		.80
4.0	.73	97.3	92.8	.53		
3.0	.53	95.0	96.0	.16	0.71	.87
		94.3	96.3	.11	.71	.82
2.5	.68	96.4	98.3	.17	.72	.89
		96.8	98.1	.20	.69	.89
2.0	.60	95.5	97.8	.12	.85	.97
		94.6	96.9	.12	.85	.97
4.5 Per Cent Gelatin.						
2.0	8.3	7.1	12.5	.01	.94	.95
2.0	6.6	16.3	23.8	.04	.92	.96
		16.0	23.5	.04	.96	1.00
2.0	3.3	47.6	53.0	.14	.79	.93
		47.3	53.4	.13	.79	.92
2.0	1.9	97.0	101.0	.21	.59	.80
		100.0	100.0	.29	.59	.88

[6] Hitchcock, D. I., *J. Gen. Physiol.*, 1923–24, vi, 95.

nitrogen groups since removing 0.40 millimols of nitrogen per gm. of gelatin decreased the hydrogen equivalent by the same amount.

The experiments were therefore repeated with deaminized gelatin with the results shown in Table II. The combining equivalent is now about 0.4 to 0.5 millimols copper per gm. of gelatin which agrees again with the figure for hydrogen.

In both of the above experiments it is difficult to prove conclusively that the figure obtained is really a maximum value since the experiments cannot be carried into higher salt concentration owing to the experimental errors. If the copper really combines with the same groups as does hydrogen, however, it should be possible to show that the sum of the two ions combined is constant, provided one or the other is present in excess. That is, adding copper to gelatin in the presence of excess acid should result in the displacement of hydrogen ion by copper, while adding acid to gelatin in the presence of excess copper should result in the liberation of copper ions, the sum of the amount of ions combined should however remain constant and equal to 0.9 millimols per liter. The results of the experiments given in Table

TABLE IV.

$AlCl_3$. 5 Per Cent Gelatin.
Gravimetric Cl Analysis. 37°C.

P.D.	Concentration of Al^{+++}			Millimols Cl combined per gm. gelatin
	Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	
	Outside	Inside		
mv.				
25.0	1.41	4.30	0.08	0.037
18.0	3.90	8.75	.16	.065
11.40	8.60	16.0	.27	.16
4.90	30.4	41.0	.47	.24
1.46	107.0*	120.0	.57	.40
1.80	74.3*	86.7	.52	.40
.60	238.0	250.0	.51	.34
.72	276.0	287.0	.62	.16
.50	484.0	495.0	.67	.10
.24	445.7	457.3	.44	.40
.08	993.0	1006.0	.45	.60

*Gravimetric Al^{+++} analysis.

III show that this prediction is carried out and confirm the conclusion that the copper and hydrogen combine with the same groups in the protein molecule. The experiments also show that there is an equilibrium between the combined Cu^{++} and H^{+}.

Chloride.—The combined chloride ion in all the experiments is less than the copper and approaches a value of about 0.3 millimols per gm. gelatin in the higher concentrations. The figure with deaminized gelatin is the same as that with gelatin. The chloride therefore combines independently of the copper. Since there is more copper

TABLE V.

$LaCl_3$.

Isoelectric Gelatin. 37°C.

Concentration of gelatin	P.D.	Concentration of La^{+++}				Millimols Cl combined per gm. gelatin
		Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	Average	
		Outside	Inside			
per cent	*mv.*					
10.0	21.6	1.2	4.2	0.04		0.039
10.0	17.85	3.55	8.83	.087		.056
1.8	1.9	10.2	12.1	.21		
1.8	1.10	36.2	39.0	.40		.23
.84	.41	36.3	37.5	.34	0.34	.20
10.0	6.84	25.4	38.7	.27		.17
1.8	.48	111.0	115.7	.57		.37
.85	.20	110.8	112.3	.47	.50	
10.0	3.86	70.1	89.5	.44		.24
10 0	.60	354.0	381.7	.49		.59

than chloride ion combined the resulting protein copper complex should have a positive charge. This was found to be the case, since gelatin in 0.1 M copper chloride migrated to the negative electrode when tested by the U-tube method using non-polarizable electrodes.

Aluminum and Lanthanum.—Loeb[7] found that La^{+++} and Al^{+++} made protein particles positive and suggested that this was due to the formation of a complex ion of the protein and the metal ion. The experiments in Tables IV and V show that both lanthanum and

[7] Loeb, J., Proteins and the theory of colloidal behavior, McGraw-Hill, New York, 1924.

aluminum do combine with gelatin to a considerable extent. The combining equivalent appears to be approaching a value of 0.5 to 0.6 millimols per gm. gelatin but in this case again it is difficult to prove that this is really a maximum value. There seems no doubt, however, that the figure is smaller than that for copper. If the aluminum and lanthanum combined with the other nitrogen groups but not with the NH_2 which are removed with nitrous acid, the combining equivalent would be about 0.5 millimols per gm. and this is the value found. In confirmation of this assumption it was found that the figure for La combined with deaminized gelatin (Table VI) was not significantly different from that for gelatin.

TABLE VI.

Deaminized Gelatin. 37°C.

Concentration of gelatin	P. D.	Concentration of La^{+++}			Millimols Cl combined per gm. gelatin
		Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	
		Outside	Inside		
per cent	*mv.*				
1.8	.67	19.91	21.1	.15	.12
		19.95	21.3	.16	.14
1.8	.32	100.2	102.0	.30	
		99.5	101.3	.31	
1.8	.14	302.0	306.7	.54	.44
		295.3	298.7	.45	.22

The chloride combined was nearly the same as that found with copper showing again that the two ions combine independently.

$CaCl_2$.—The results with $CaCl_2$ are given in Table VII. The combined calcium approaches the value of 0.9 millimols per gm. of gelatin as in the case of copper but the maximum is not reached until a much higher concentration of salt. The value with deaminized gelatin at pH 5.0 is the same as for gelatin. Apparently the Ca^{++} therefore does not combine with the NH_2 groups but with the carboxyl groups. The carboxyl group equivalent of gelatin is also about 0.9 millimols per liter.

Magnesium Chloride.—Table VIII gives the results of the experiments with $MgCl_2$. The combined magnesium approaches 0.4 – 0.5 millimols per gm. as in the case of Al and La. This low figure is probably due to the fact that the solutions are slightly acid. The combination with deaminized gelatin is about the same or higher, at

TABLE VII.

$CaCl_2$.

1.6 Per Cent Gelatin. pH 5.0.

P.D.	Concentration of Ca^{++} Millimols per 1000 gm. H_2O		Ca^{++} Millimols combined per gm. gelatin
	Outside	Inside	
mv.			
0.20	296.0	300.8	0.58
	294.0	297.2	.49
.10	496.0	506.0	.78
	492.0	496.0	.47
.10	751.0	758.0	.70
	738.0	744.0	.63
.13	982.0	993.0	1.2
	986.0	996.0	1.1
.36	882.0	914.0	.77
(7.3 per cent gelatin)	916 0	954 0	.83
Deaminized Gelatin pH 5.0 + $Ca(OH)_2$.			
.09	948.0	957.0	1.00
	962.0	968.0	.87
.15	1008.0	1034.0	.90
(4.4 per cent gelatin)	1008.0	1035.0	.89

pH 5.0. $CaCl_2$ also gave low results when the solution was not kept at pH 5.0 by the addition of alkali. In the case of $MgCl_2$ this cannot be done owing to the insolubility of $Mg(OH)_2$.

KCl, NaCl, LiCl.—In all the former experiments the membrane potential has been positive showing that the non-diffusible ion was positive. In the case of KCl however the membrane potential is

negative and it is found that the amount of K combined is within the error of the method. In fact the value comes out very slightly negative. The combined Cl is also less than in the other experiments but is still enough to be significant. The experimental results are shown in Table IX. There is therefore a qualitative difference between KCl

TABLE VIII.

$MgCl_2$.

10 Per Cent Gelatin. 37°C.

P.D.	Concentration of Mg^{++} Millimols per 1000 gm. H_2O Outside	Concentration of Mg^{++} Millimols per 1000 gm. H_2O Inside	Concentration of Mg^{++} Millimols combined per gm. gelatin	Concentration of Cl^- Millimols combined per gm. gelatin
mv.				
1.51	15.0	18.0	0.04	0.04
.94	62.0	68.1	.10	.07
.20	569.0	582.0	.21	.14
.30	1027.0	1042.0	.36	.07
.40	1140.0	1151.0	.47	
.30	964.5	982.5	.39	.15
.23	2107.0	2131.0	.60	.12
Deaminized Gelatin. 1.7 Per Cent. pH 4.1.				
.11	303.0	303.8	.18	
	303.0	303.8	.18	
.16	501.5	502.5	.38	−.1
	500.0	501.0	.35	−.2
Deaminized Gelatin at pH 4.7 (+ NaOH).				
.04	298.2	306.0	.49	
	292.8	300.2	.46	
.16	492.0	502.0	.89	
	494.0	504.5	.94	

and the di- and trivalent ions studied, in that KCl forms a negative complex ion while the other salts form a positive complex ion. In any system therefore which requires a very small or no potential there would be a sharp antagonism between the effect of Na, K and Li ions and Mg or Ca ions.

$AgNO_3$.—The results for $AgNO_3$ are given in Table X. As would be expected, Ag^+ is combined more than the other monovalent ions. The nitrate ion was not combined.

Na_2SO_4.—No combination of either ion was found and no potential was obtained.

TABLE IX.

KCl.

10 Per Cent Gelatin. 37°C.

P.D.	Concentration of K			Cl
	Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	Millimols combined per gm. gelatin
	Outside	Inside		
mv.				
−0.18	98.0	96.8	−0 02	0.1
	98.3	98.8	− .02	.1
− .05	499.4	497.8	− .02	
	497.8	496.8	− .01	.05

TABLE X.

$AgNO_3$.

5 Per Cent Gelation. 37°C.

P.D.	Ag^+			NO_3^-
	Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	Millimols combined per gm. gelatin
	Outside	Inside		
mv.				
3.12	17.4	20.9	0.10	0 02

TABLE XI.

Na_2SO_4. 1.8 Per Cent Gelatin.

pH	P.D.	Concentration of $SO_4^{=}$ Millimols per 1000 gm. H_2O	
		Outside	Inside
	mv.		
4.7	0.2	96.8	96.9

Effect of the Hydrogen Ion Concentration.—The foregoing experiments were all done as nearly as possible at pH 4.7, with the exception of those in which acid was added to the copper. It was found in that case that the copper was displaced by the addition of acid. In the case of copper it is not possible to work on the alkaline side so that it seemed of interest to complete the experiment with another ion.

The results of a series of experiments with 0.1 M $CaCl_2$ at various pH values are given in Table XII. As in the case of copper the addition of acid quickly replaces the calcium so that on the acid side of

TABLE XII.

HCl. $CaCl_2$.

1.8 Per Cent Gelatin.

pH	P.D.	Concentration of Ca^{++} Millimols per 1000 gm. H_2O		Ca^{++} Millimols combined per gm. gelatin
		Outside	Inside	
	mv.			
2.5	0.50	100.0	96.2	
3.4	.3	101.5	99.0	
3.8	.3	98.6	98.2	0.10
4.1	.5	100.0	99.5	.19
4.7	.4	98.0	100.0	.29
5.4	.10	96.5	98.5	.15
7.0	.2	100.6	103.8	.29
8.6	.12	98.8	102.9	.26
10.0	.24	99.4	105.0	.35
		99.7	104.4	.41

pH 3 no combined calcium was found. The value rises rapidly as the solution becomes less acid and remains approximately constant on the alkaline side of pH 4.7.

SUMMARY.

1. The combination of Cu^{++}, Ca^{++}, Mg^{++}, Al^{+++}, La^{+++}, K^{+}, Ag^{+}, and Cl^{-} with gelatin has been determined.

2. The equivalent combining value for copper is about 0.9 millimols per gm. of gelatin and is therefore the same as that of hydrogen. The value for copper with deaminized gelatin is about 0.4 to 0.5, again the

same as that of hydrogen. The sum of the hydrogen and copper ions combined in the presence of an excess of either is 0.9 millimols showing that there is an equilibrium between the copper hydrogen and gelatin and that the copper and hydrogen are attached to the same group.

3. The equivalent combining value of La^{+++} and Al^{+++} is about 0.5 millimols per gm. of gelatin. This value is not significantly different with deaminized gelatin so that it is possible these salts combine only with groups not affected by deaminization.

4. No calcium is combined on the acid side of pH 3. The value rises rapidly from pH 3 to 4.7 and then remains constant.

5. No combination of K, Li, Na, NO_3 or SO_4 could be detected.

6. Cl combines less than the di- and trivalent metals so that the protein is positive in $CaCl_2$ but negative in KCl.

THE VAPOR PRESSURE OF DOG'S BLOOD AT BODY TEMPERATURE.

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I.

INTRODUCTION.

An evaluation of the *colligative* properties of the blood is of paramount theoretical importance, for such properties enter as fundamental factors in all physicochemical considerations based either on the kinetic theory or on thermodynamics. Despite this importance, no direct measurements of the colligative properties of blood have been made at body temperature.[1] Our knowledge of these functions is based almost exclusively on freezing point determinations made at 0°. However, the importance of the colloidal constituents of the blood and the magnitude of the unknown change which temperature may produce in their properties renders the application of such measurements to conditions obtaining in the intact organism rather hazardous. Because of the lack of a direct determination of any of the colligative properties of blood at body temperature, it was deemed worth while to make such a measurement.

To obtain a value for the colligative properties of a solution four possible methods are principally used, *viz.* the determination of the raising of the boiling point, the lowering of the freezing point, the osmotic pressure, and the vapor pressure. The first of these methods is impossible in the case of blood solutions. Objections to applying freezing point data to blood at body temperature have already been

[1] Friedenthal (1) attempted to obtain such data by using the tensimetric method for determining vapor pressure. This method involves the evacuation of the system under investigation, a process objectionable in the study of blood. Moreover, it is not capable of yielding a high degree of accuracy.

noted. We are thus restricted to direct osmotic pressure measurements or to the determination of the vapor pressure, in order to obtain exact data concerning the condition of the blood in the state in which we are interested in its manifestations—*viz.* at body temperature. Direct osmotic pressure measurements are beset with many experimental difficulties and pitfalls which often render the results of doubtful value. This is particularly true of attempts to measure the osmotic pressure of solutions of electrolytes as has been elsewhere discussed (2). The recently described method of Frazer and Patrick (3), assuming its validity, would be inapplicable to blood solutions, since it requires evacuation to remove all dissolved gases, which are essential constituents of normal blood.

The determination of the vapor pressure is, therefore, the only remaining method applicable to blood at body temperature. This method was consequently used in this investigation. As we shall see later, relationships exist by which the other colligative properties may in turn be calculated from vapor pressure data. The most exact vapor pressure measurements which have been made by the static method are those of Frazer, Lovelace, and their associates (4). The necessity of having gas-free solutions, obtained by long boiling and repeated evacuations, makes this method inapplicable to blood solutions. The original dynamic method of Ostwald and Walker (5) has also been modified, to give very exact results, by Berkley, Hartley, and Burton (6) and by Washburn, Gordon, and Heuse (7). In the dynamic method it is possible to maintain the blood under the same conditions as exist in the organism. The necessity of passing a stream of gas over the solution to be investigated permits the use of a gas of the composition of the *alveolar* air, thus maintaining the blood in equilibrium with the same gaseous mixture with which it normally comes into equilibrium in the body. For the purpose of the present investigation, therefore, the dynamic method as modified by Washburn and Heuse (7) was followed.

II.

EXPERIMENTAL.

In the dynamic method, as used here, a large volume of air is passed over the solution investigated with which it comes into equilibrium. The gas saturated

with the vapor from the solution is then led over an absorber which removes the water vapor. The dried gas is next led through a similar train in series with the first where it becomes saturated with water vapor from pure distilled water and it then gives up this water vapor to a second absorber. By weighing the absorbers before and after the experiment, a differential measure of the vapor pressure of the solution as compared with pure water, whose vapor pressure at the temperature of the experiment is known, can be obtained.

To simulate as closely as possible the conditions obtaining in the animal body, a gas mixture approximating the composition of the alveolar air with which the arterial blood of the body is in equilibrium was used.[2] This was obtained by forcing CO_2 under pressure into a steel cylinder containing compressed air. This gas was led through a needle valve to a coil of about 20 feet of copper tubing, contained in the thermostat, where the gas assumed the temperature of the thermostat, before entering the saturators. The saturators were of the type described by Washburn and Heuse (7). They consisted of a train of glass tubes placed on a rocking table. The other parts of the apparatus were mounted on shelves of the same rocking table and the whole was rocked to and fro at a rate of ten per minute. The saturators were about half filled with liquid and the movement of the rocker stirred their contents, thus facilitating saturation of the gas with the vapor. The apparatus was kept in a water bath maintained at 37.50° $\pm$.05° by the usual methods of thermostatic control.

The blood used in these experiments was obtained by bleeding normal mongrel dogs. In order not to introduce any foreign substances in the form of anesthetics into the blood stream, a mode of local anesthesia, for which I am indebted to Professor E. K. Marshall, Jr., was employed. This consisted in infiltrating the region of the femoral artery with distilled water, which procedure combined with freezing with ethyl chloride permitted cannulation of the artery and bleeding of the animal to death. In this way about 1 liter of blood could be obtained from a 15 kilo dog, an amount sufficient for several determinations.

To determine the nature of the blood obtained in the above procedure, the following experiment was performed. A mongrel weighing 14.00 kilos was bled as described. The blood was collected in five successive vessels containing heparin. The total amount collected in this way was 855 cc. An additional quantity measuring 105 cc. was obtained by opening the chest and emptying the heart and large vessels of the thorax. Thus a total of 6.8 per cent of the body weight of the dog constituted the minimal blood volume. Even assuming a conservative estimate of the blood remaining in the tissues, one obtains a figure

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[2] The importance of this procedure is illustrated by the work of Kovács (8) who showed that aeration of blood with CO_2 caused an increase in the value of its freezing point lowering while oxygenation of the blood, both *in vivo* and *in vitro* caused a decrease in the value of the freezing point lowering. Similar findings are reported by Bottazzi (9).

indicating the accuracy of the higher values obtained for the blood volumes of the dog (25). The corpuscular volume, the red blood cell count, the hemoglobin, and the protein content of the plasma, were determined in fractions of the collected blood. The corpuscular volume of the first fraction of blood collected, as determined by the hematocrit, was 48.5 per cent as compared with the value 47.5 per cent obtained on the last fraction of blood obtained. The red blood cell counts of these two samples likewise differed by only 3 per cent. The *hemoglobin* content of the last collected sample, as determined by the acid hematin method against that of the first collected sample was 97 per cent. The *protein* content of the plasma of the blood last collected was 99 per cent of that first obtained, as determined by the refractometric method. It is thus obvious that in the method involving the use of a local anesthetic, no appreciable dilution (greater than 2 or 3 per cent) of the blood occurs in bleeding an animal to death through a large femoral cannula. The rapidity with which the bleeding takes place, no doubt, does not permit the passage of much fluid from the tissues into the blood with its consequent dilution.[3] The blood obtained by this method may therefore be considered as representative of the mixed total blood of the animal.

To prevent coagulation, *heparin* was added; and to lessen the rapidity of decomposition by bacterial invasion, *acriflavine* was added. An equal amount of heparin and acriflavine was also added to the distilled water against which the vapor pressure of the blood was determined, to counterbalance the minimal lowerings which these substances produced. The decomposition of blood at 37.5° is one of the most serious difficulties which one encounters in experimental procedures prolonged over many hours as in the case of these experiments. An aseptic technique is difficult to attain. Bacterial decomposition of blood takes place very rapidly with a rapid increase in the freezing point lowering, as shown in the experiments of Carrara (10). The addition of some preservative such as acriflavine is, therefore, essential to avoid the erroneous and fantastically high results otherwise obtained. Another serious difficulty encountered in the study of blood which does not enter into vapor pressure studies of simple solutions is the tendency of blood to foam. Although the aerating gas in the method here employed does not pass through the blood, but merely over it, this tendency for the blood to foam persists. A bubble carried over to the absorbers would naturally give a very high result. Traps were therefore inserted in the system to prevent this. When the rocking mechanism was so adjusted as to work with least jarring the frothing was minimal.

The possibility of obtaining supersaturation of the gas as a result of frothing and the effect of decomposition are two factors which render the accuracy of this method when applied to blood much less than that obtained when simple solutions

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[3] This finding is contrary to the conceptions found in the current text-books (*cf.* Starling, E. H., Principles of human physiology, Philadelphia, 4th edition, 1926, 866).

are used. Test runs with water in the saturators are therefore deceiving as regards the ultimate accuracy obtained. Thus Washburn and Heuse (7) obtained an average deviation of only 0.4 per cent in the value of the relative vapor pressure lowering of a molar sucrose solution. No such high degree of accuracy is obtained in similar experiments on blood solutions.

III.

EXPERIMENTAL RESULTS.

The results obtained on blood plasma are given in Table I. The plasma was prepared by centrifuging heparinized blood obtained as described above. The plasma thus obtained was always perfectly clear and was free of the hemolysis so commonly obtained from dog's blood that has been defibrinated.

In the first column of Table I are given the experimentally determined vapor pressures, which are calculated in the following manner (7). If a volume of gas, v, is equilibrated with a liquid, whose vapor pressure is p, the mass of water vapor, m, taken up by the gas is given by the relation:

$$m = K\,p\,v$$

where K is a constant. The ratio of the masses of water absorbed from two absorbers will be

$$\frac{m_1}{m_2} = \frac{p_1 v_1}{p_2 v_2}. \qquad (1)$$

If the same volume of gas passes over two liquids, the volumes v_1 and v_2 respectively emerging from the saturators containing the liquids are inversely proportional to the partial pressures of the air in the saturators, *i.e.*,

$$\frac{v_1}{v_2} = \frac{p_2}{p_1},$$

where p_2 and p_1, the partial pressures of the gas in the saturators, are equal to the barometric pressure minus the vapor pressure of the water in the saturator minus the difference between the barometric pressure and the pressure within the saturator, *i.e.*,

$$\frac{v_1}{v_2} = \frac{B - p_2 - \Delta\,p_2}{B - p_1 - \Delta\,p_1}, \qquad (2)$$

where B is the barometric pressure and Δp_2 and Δp_1 are the differences obtained by a manometer connected to the exit tube of the suturator and open to the atmosphere. Combining equations (1) and (2) gives

$$p_1 = \frac{m_1 p_2(B - \Delta p_1)}{m_0 (B - p_2 - \Delta p_2) + m p_2}. \quad (3)$$

By placing water in one saturator and the solution to be investigated in another, one can determine the vapor pressure of the latter from the weights of the water absorbed from each saturator, the barometric pressure, and the difference in pressures between the interior of the saturators and the atmosphere. Throughout this paper the vapor pressure of pure water at 37.5° is taken as 48.38 mm. of mercury,[4] as found by Heuse and Scheel (11).

TABLE I.

The Colligative Properties of the Blood Plasma of the Dog.

I Vapor pressure observed	II Freezing point lowering observed	III Vapor pressure calculated from freezing point data	IV Osmotic pressure at 37.5°C. calculated from the vapor pressures observed
mm.Hg	*°C.*	*mm.Hg*	*atmospheres*
48.09	0.616	48.09	8.5
48.12	0.605	48.10	7.6
48.11	0.604	48.10	7.9
48.08	0.620	48.09	8.8

IV.

Calculation of the Vapor Pressure Lowering from Freezing Point Values.

Since the great mass of existent data on the osmotic pressures of physiological solutions are based on freezing point measurements, determinations of the latter were also made by the usual Beckmann technique on all of the solutions studied. These values are recorded in Column II of Table I. It is of interest and importance, moreover, to compare the vapor pressures as calculated from freezing point data with those obtained by direct measurement.

One may calculate the vapor pressures of solutions from freezing

[4] Holborn, Scheel, and Henning (23) give the value 48.36.

point data by the use either of Callendar's equations (12) or that of Washburn (13). The latter's equation is

$$\log_{10} \frac{p}{p_0} = \frac{\Delta C_p}{R} \log_{10} \frac{T_F}{T_{F_0}} - \frac{0.4343\ (L_{F_0} - \Delta C_p\ \Delta T_F)}{RT_F} + \frac{0.4343\ L_{F_0}}{RT_{F_0}}.$$

In this equation ΔC_p is the difference in the molar heat capacities of the solvent in the liquid and solid states; R is the gas constant; L_{F_0} is the molar heat of fusion of the pure solvent at the freezing point of the solvent; T_{F_0} is the absolute freezing point of the solvent; T_F is the absolute freezing point of the solution; ΔT_F is the lowering of the freezing point and p and p_0 are the vapor pressures of solution and solvent respectively. The values of ΔC_p, R, L_{F_0}, and T_{F_0} are 9.04, 1.985, 1435.5, and 273.1, respectively. When ΔT_F is less than 1.0, as in the case of blood solutions, one may with sufficient accuracy utilize the simplified equation proposed by Callendar

$$\log_e \frac{p_0}{p} = \frac{L_{F_0}\ \Delta T_F}{RT_0^2} = 0.009696\ \Delta T_F.$$

The calculations of the vapor pressure of blood from the freezing point determinations are given in Column III. It will be seen that the agreement between the experimental and calculated vapor pressures are as good as the experimental errors involved allow. In calculating vapor pressures from freezing point data for temperatures other than the freezing point, as is done here, there are two factors which are neglected *viz.*: the change in the heat of dilution of the solution with temperature and the change in the degree of dissociation with temperature. In so far as sodium chloride, the chief electrolytic constituent of the blood, is concerned, however, these factors would not be expected to greatly influence the results of our calculation. Thus a comparison of the highly accurate vapor pressure data of aqueous sodium chloride solutions at 20° as measured by Norris (14) with values calculated by Callendar's simplified equation from the freezing point data of Jahn (15) and Rodebush (16) show perfect agreement up to concentrations of 1 M. The results may therefore be taken to indicate that the freezing point determinations of plasma may be considered as at least fairly accurate measurements of the true colligative properties at body temperature.

V.

Calculation of the Osmotic Pressure from the Vapor Pressure Data.

Recent developments in the application of thermodynamics to solutions have reduced the important status which was previously assigned to osmotic pressures. Its place has been taken by a much more useful and generally applicable function, the *activity*. For physiological purposes, however, the concept of osmotic pressure remains of paramount importance, for it is the chief and most tangible force associated with the movement of fluids within the organism. Moreover, the complexity—as regards the number of constituents—and the paucity of our knowledge, render application of the concept of activity to blood impossible at present.

To calculate the osmotic pressures from the vapor pressure data obtained in this investigation we utilize the thermodynamically derived relation (17),

$$P = \frac{RT}{V_0} \log_e \frac{p_0}{p}.$$

In this equation P is the osmotic pressure at the absolute temperature T; V_o is the molecular volume of the solvent under the standard pressure; p_o is the vapor pressure of the solvent; p is the vapor pressure of the solution; and R is the gas constant. This equation neglects the compressibility of the solution and assumes that the vapor follows the gas laws. In the case of blood solutions, the experimental accuracy would not warrant consideration of these factors which, moreover, are very small and hence may be neglected. Substituting 82.07 for R, 310.6 for T, 18.016 for V_o, and converting to Briggsian logarithms, the above equation becomes

$$P = 3258 \log_{10} \frac{p_0}{p_0}.$$

The osmotic pressures in atmospheres calculated by means of this equation from the experimental vapor pressure determinations are given in Column IV, Tables I and II.

VI.

The Vapor Pressure of Dog's Blood.

The results obtained with dog's blood are given in Table II. Calculations similar to those described for plasma were also carried out for the whole blood and are recorded in the table. In general, the vapor pressures of the whole blood are seen to be the same as that of plasma.

Collip (18) found that the lowering of the freezing point of the blood corpuscles of various species was always less (from 0.02° to 0.07°) than that of the serum. Whole blood, likewise, gave a lower freezing point lowering than the plasma, as had previously also been found by other workers (19). This phenomenon, if real, is rather difficult to explain. Assuming permeability of the corpuscular cell wall to water, the activity of the water within the cell and that of the plasma must be the same when equilibrium is attained. Conversely, the freezing points of the cells and plasma must be the same. It would therefore seem that determinations of the freezing point of such systems as blood cells or of whole blood are in error, and that the existence of the two phase system *ice-solution*, in the case of the red corpuscle, is not clearly recorded by the ordinary methods for determining freezing points. On the other hand, it is possible that the aqueous phase within the corpuscle is not comparable to that in the plasma. In other words we have to deal with a two phase system analogous to that of the system—*water saturated with phenol* and *phenol saturated with water*. Such a view is quite contrary to the usually accepted data concerning the equilibrium between corpuscles and plasma (20).

TABLE II.

The Colligative Properties of Dogs' Whole Blood.

Vapor pressure observed	Freezing point lowering observed	Vapor pressure calculated from freezing point data	Osmotic pressure at 37.5°C calculated from the vapor pressures observed
mm.Hg	°*C.*	*mm.Hg*	*atmospheres*
48.08	0.602	48.10	8.8
48 12	0.585	48.11	7.6
48.09	0.590	48.11	8.5

As far as vapor pressure measurements are concerned, however, it must be remembered that the observed vapor pressure from an heterogeneous system will always be that of the more volatile phase.

VII.

The Vapor Pressure Lowering of Blood Plasma Compared to That of Its Ultrafiltrate.

It is the concensus of opinion that the colloids of blood plasma exert only a minimal osmotic pressure compared to that of the other constituents. Direct determinations of the osmotic pressure of plasma measured against the ultrafiltrate of the plasma give values between 30 and 50 mm. of mercury (21). The difficulty attendant upon such direct measurements and the assumptions frequently made concerning the existence of a high degree of water binding by the blood colloids made a redetermination of the osmotic pressure of the blood by some new and direct method seem worthy of further investigation. Neuhausen (22) attempted the solution of this problem by the original Ostwald-Walker method (5). In the present investigation, blood plasma, obtained as already described, was ultrafiltered according to the technique and with the precautions previously described (24) as essential for obtaining true ultrafiltrates from colloidal solutions. Such ultrafiltrates from blood plasma were put in the saturators previously occupied by distilled water and their vapor pressure determined against that of the plasma part of which had been used in preparing the ultrafiltrates. The average of three such determinations gave a vapor pressure value corresponding to about 0.06 atmosphere for the osmotic pressure of the blood colloids. This value will be seen to be in accord, so far as the experimental accuracy permits one to judge, with the commonly accepted values for the osmotic pressure of the blood colloids. An exact evaluation of this function can be obtained only by direct osmotic pressure determination.

VIII.

SUMMARY.

The vapor pressures of dog's blood and blood plasma were determined at 37.5° by the dynamic method and the osmotic pressures

calculated from the experimental data. The vapor pressures calculated from experimentally determined freezing point data agreed, within the experimental error, with the values obtained from direct measurement. The vapor pressure lowering produced by the colloid constituents of the blood was also determined and found to be minimal compared to that of the other constituents.

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DRUG ACTION IN GALVANOTROPIC RESPONSES.

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The responses of nemertean and gephyrean worms to galvanic currents, before and after treatment with drugs, have been taken (Crozier, 1926–27) to indicate that the primary action of strychnine in reversal effects has a locus which is not perikaryal, but is presumably synaptic. It was desirable to determine if unrelated forms would yield similar evidence.

The trough used for most of the observations on the galvanotropic responses of the animals which I have tested was made of "parawax," but one of glass served for the final experiments on *Planaria maculata*. Both were rectangular, 5 cm. wide, and 20 cm. long. Constant electric current derived from a 110 volt circuit passed through 1 cm. depth of tap water in the trough between "nonpolarizable" electrodes (*i.e.*, pads of absorbent cotton). The total effective current could be easily and gradually varied, by means of a rheostat, from 0 to 3 ma. or more, although 0.3 – 0.8 ma. (giving a current density of 0.06 – 0.16 ma. per sq. cm.) generally proved optimal; the potential was less than 5 volts with all the currents tried.

Isopods.—The effects of strychnine and of other neurophil drugs in relation to galvanotropism seemed of especial interest in arthropods, since their nervous system has revealed properties different from those characterizing other invertebrates (Crozier, 1922; Crozier and Pilz, 1924). Moreover, the anodic behavior of normal individuals of the fresh water isopod *Asellus communis* (Say) exposed to galvanic currents seemed favorable for these experiments.

If a current of moderate intensity (about 0.5 ma., or 0.1 ma. per sq. cm.) is switched on, a resting *Asellus* reacts sharply, usually starting forward if facing the anode, backward if facing the cathode, and at the same time usually turning the head end away from the cathode; if transverse to the current, the animal moves sidewise toward the anode and some evidence of U formation may appear, both anterior and posterior ends bending toward the anode, despite the creature's relative shortness and rigidity. Most often the animal will be more or less oblique

to the lines of current flow and the movements then elicited are corresponding resultants of the foregoing. Clearer orientation away from the cathode, as usual not completely toward the anode, resulted also and in the first instance from the oblique position. A swimming animal is halted on the make of an ascending current (*i.e.*, flowing postero-anteriorly) but not of a descending current. While weaker currents are simply less efficacious in prompting these responses (especially the sidling to the anode), stronger currents obscure them by causing ventral flexion of the head away from the anode and backward creeping toward the cathode. If the current is gradually increased from 0 to 0.8 ma., the anodal character of the resulting behavior remains as when the current is suddenly established, sidling (of transverse animals) towards the anode appearing with about 0.4 ma.; but sharp movements of any sort rarely occur in the absence of the make shock, which sometimes, however, acts just as touch to stimulate "death feigning" immobilization.[1]

When a few of these isopods, selected as active and showing typical anodal movements, were left immersed in strychine sulfate solutions (in tap water, 1:20,000 and 1:10,000), there was apparent in 18 hours no effect further than a possible tendency to ventral flexion of the head and to a drawing together of the legs. Also, after 3 days in the stronger solution, followed by removal to tap water, one individual was abnormally sluggish, tending to lie on its back, and showed poor coordination of the legs when prodded into creeping. Solutions of the strychnine base (1:10,000) had little effect other than to induce, after at least an hour's exposure, convulsive ventral flexures and entangling adductions of the legs from time to time; the animals acted normally between the short convulsions, running when prodded, and 2 days later, on a second trial, were even less affected. In the case of saturated solution, increased sensitivity to touch suggested itself after a few minutes only; after 1 to 18 hours, the only discernible effect was some disorganization of locomotion, with ventral flexure. In short, strychnine did not cause reversal of the reciprocal inhibition involved in normal movements, or other pronounced effects, in *Asellus*. The strychninized animals were, besides, anodic as normally, aside from the threshold being raised after several hours in the solution.

Atropine sulfate (in solution of 1:1000) failed to induce any discernible departure from the normal behavior or from the anodal galvanotropism of animals which

[1] A single individual, among the fairly large number examined, on first being taken from the aquarium in which all the stock was kept for a number of weeks, and again after 2 hours, exhibited at the make of 0.6 ma. responses of the sort described, except toward the cathode instead of the anode. When next tested, on the same day, this individual made the normal *anodal* movements in the same current, and no subsequent reversal was seen in the 2 days that it survived (while others lived weeks longer under similar conditions). This is of interest as it shows that galvanotropic reversal is possible in *Asellus*. The "death feigning" reactions, obtainable in all, seemed more frequent and of longer duration in animals which died within a few days.

were immersed in it for 48 hours, either during that period or within 3 subsequent days. As many as 42 hours in caffeine (1:10,000) yielded similar, negative results.

With nicotine (1:50,000) marked changes from normal posture and movements appeared in from 10 minutes to 3 hours. Animals immersed longer (as much as 7 hours) showed increased immobility and cramped attitudes involving now dorsal flexure of a whole animal, with or without raising and spreading of the legs, and now the reverse; at the same time, the antennæ were folded together anteriorly or spread stiffly back along the body, and the leg movements were disorganized, or if creeping could be induced by prodding from behind it was generally reversed, namely backwards, progress forward seeming impossible. These effects appeared to recur periodically, for at intervals the behavior was practically normal, including even forward creeping on stimulation. Numerous trials, made in the course of the nicotinization, failed to reveal either reversal or exaggeration of the typical anodal galvanotropic responses. When the just described nicotine effects were more evident, significant movements could not be elicited with up to two or three times the usually effective current. On the other hand, when the usual or the stronger currents were effective, the responses were anodal as before and remained such for the several days during which the nicotine effects were still observable.

The practical ineffectiveness of strychnine as concerns *Asellus* agrees with previous findings for other arthropods (Crozier and Pilz, 1924), which may also be said for the absence of any reversal of inhibition (Crozier, 1922). Although impermeability of the exoskeleton may be a factor in the failure of the isopods to show any reaction to atropine and caffeine, clearly nicotine, at least, can penetrate. Observations by Crozier (1922) indicate that in caterpillars an effect of nicotine may be to induce reversal of certain motor responses to touch, as appeared in *Asellus*. The galvanic current effects, however, threw no further light on the nature of the action of nicotine on different or related invertebrates (*cf.* Crozier, 1926–27[2]).

Planarians.—Planaria maculata (Leidy) not only forms a U and orients toward the cathode (Hyman and Bellamy, 1922), but, consistently with Loeb's (1918) explanation of galvanotropism (*cf.* Moore, 1922–23), shows slightly strained, differential contraction of the longitudinal and the circular muscles, by lengthening (if not already elongated) and perhaps moving forward when facing the cathode, and by stopping and shortening when facing the anode. A total current of 0.3 to 0.5 ma., established either abruptly or gradually,

[2] Crozier (1926–27), p. 405.

generally sufficed to induce essentially these same responses in *P. agilis* (Stringer) and *P. velata* (Stringer), as likewise in *P. maculata,* and increased current density did not alter their cathodic character. Most of these experiments were on *P. agilis,* but *P. maculata* also figured, and decisively, in those with strychnine.

Whether the strychnine used be a 1:10,000 solution of the base or of the sulfate, the planarians begin in 3 minutes of immersion to elongate excessively and to twist about convulsively rather than creep. While remaining longer in the solution, or for some time after removal (from 10 or more minutes immersion) into tap water, the worms exhibit reversal of inhibition in that they elongate markedly or remain extended in response to touch, which normally, applied to the anterior half, elicits at least initial retraction of the head, if not complete shortening, of an active individual (*cf.* Moore, 1918–19).

Under these conditions, the absence of coordinated locomotor activity and the persistence of coiled and spiral elongation make it difficult to determine galvanotropic orientation. Strychninized planarians fail to show the usual cathodal responses to the ordinary or to increased current strengths; the latter, indeed, tend to set off more convulsions. The lengthening and shortening responses, however, are certainly reversed though trebled current may be required, while such bending as may become evident is (reversed) toward the anode. Although the whole animals do not uncoil so far that they can be placed entirely parallel to the current, they elongate still further when the head end is nearer the anode and *vice versa.* These responses are more readily distinguished if the curvature be lessened by cutting the animals in two. Both parts, but especially a head portion extending about to the mouth (and best in *P. maculata*), then elongate or shorten in currents which bring about the opposite effects in corresponding unstrychninized parts. The drugged head portions may even be manœuvred into nearly natural positions on the substrate and into locomotion. Putting such portions into the trough together with untreated but similarly amputated pieces provides an excellent demonstration of the opposite responses to the same currents. The strychninized "heads" progress, lengthened, toward the anode as the unstrychninized "heads" stop and shorten; or as the latter progress, lengthened, toward the cathode, the former stop and

shorten. Furthermore, recovery occurs within a very few hours after limited strychninization, so that all the reversal phenomena disappear; *thereupon another strychnine treatment will reinduce* them. One may add that when strong ascending currents cause some lengthwise contraction in the normal whole or part planarian, it appears in a retraction of the anodal end, which also is the end most affected by descending currents; upon strychninization, on the other hand, it is the cathodal end which first is drawn in.[3] Evidently the action of strychnine and of the galvanic current cannot both be to excite the same nervous elements. Also, in view of the responses exhibited by the caudal halves of the bisected planarians, the affected structures cannot be exclusively localized in their "heads."

The specificity of the strychnine effect becomes clear by comparison with the action of nicotine and atropine, neither of which bring about similar reversal of inhibition or reversed galvanotropism in planarians. About 1 minute in a 1:50,000 solution of nicotine is enough to cause convulsive twisting, lengthening, and shortening. In a few more minutes, however, the planarians tend more and more to remain very shortened. The galvanotropic responses continue to be cathodal, although less obvious, even with increased current, on account of the animals' sluggishness and rounded-up posture. The latter effects of nicotine agree with Rico's (1926) observations on *Ascaris*.

Atropine sulfate (1:1000) *acts in the first instance to excite the* planarians to rapid creeping in a rather elongated state. This effect appears within 5 minutes. After about 20 minutes the animals become sluggish and, between periods of activity and occasional writhing, rest in a shortened condition which, since they are pointed at head and tail, differs from that induced by nicotine. In neither the excited nor the sluggish state does a change occur from the normal cathodal responses, except that in some cases the latter appear enhanced after the immersion in the drug solution has lasted at least 40 minutes.

Oligochætes.—Some years ago Knowlton and Moore (1917) reported reversal of inhibition by strychnine in the earthworm. When the

[3] Since it is hardly possible to judge if the direction of the (strong) currents is an influence in any dorsoventrally differential, longitudinal contraction, strychnine cannot be observed to enhance such effects, but does the contrary, as it seemed to me.

typical effects are in evidence (as after the worm has been immersed in a 1:8000 solution of strychnine sulfate for 2 hours), the normal cathodal responses to the galvanic current (Moore, 1922–23) are also reversed, though about eight times the usual current of 0.3 ma. may be required to evoke them.[4] *Allolobophora fœtida* (Savigny), especially its cephalic portion, turns and *elongates* toward the anode and *shortens* and bends from the cathode, instead of contrariwise. Meanwhile the caudal end contracts away from the anode, or, if in a transverse position, may bend in conjunction with the head toward the anode to form a U, in both respects likewise showing reversal. The anodal responses may be seen, too, in any group of segments cut from a strychninized worm.

Since a given movement of a planarian or an earthworm, whether induced by a drug or by a galvanic current, may be taken to depend upon the same nerve-muscle units, the strychnine reversal of galvanotropism in these forms bespeaks, in agreement with the conclusions based on nemerteans and gephyreans (Crozier, 1926–27), a localization of the strychnine effect primarily outside the nerve cell bodies. In fact, the results obtained with the planarians are evidence for the existence in their nervous system of structures homologous to synapses.

SUMMARY.

Under strychninization involving reversal of reciprocal inhibition of the circular and longitudinal muscles, planarians and earthworms show reversed, *i.e.*, anodal, galvanotropic responses, which neither nicotine nor atropine induce (in planarians). The results strengthen the conclusion (Crozier, 1926–27) that in causing reversal of inhibition strychnine acts primarily on central synapses or on homologous elements.

Like other arthropods, on the other hand, *Asellus* is little affected by strychnine; no reversal occurs. Caffeine and atropine are even less effective. Nicotine evokes abnormal posture and movements, perhaps reversed, but no alteration of anodal galvanotropism.

[4] Recognition for the initial demonstration of this result is due to Mr. V. C. Harnish, working in this Laboratory.

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THE ANTAGONISM BETWEEN ACETIC ACID AND THE CHLORIDES OF SODIUM, POTASSIUM, AND CALCIUM AS MANIFESTED IN DEVELOPING FUNDULUS EMBRYOS.

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Loeb in his experiments (1, 2, 3) on acid-salt antagonism found that the embryos of developing *Fundulus* eggs immersed in acetic acid (M/500) were soon killed, and that the addition of certain amounts of any one of several salts served to protect the embryos from the toxic action of the acid. Loeb considered that the antagonism of the acid by the salt took place at the chorionic membrane; that the presence of the salt prevented or slowed the penetration of the acid through this membrane. He cites no control experiments on embryos of the same age from which the chorionic membranes were removed. In this paper are described the results of such control experiments together with one additional experiment. They materially modify the interpretation of Loeb's original experiments and especially his ideas as to the site and mechanism of the antagonism and its relation to permeability.

Material and Methods.

Embryos of *Fundulus heteroclitus* 5 to 8 days old were used in the experiments. By this stage the blastopore has closed and none of the body orifices have broken through so the entire external surface of the embryo is a continuous intact layer of ectoderm. The chorionic membrane which surrounds the embryo was removed under a binocular dissecting microscope with dissecting needles and iridectomy scissors. In removing the membrane, special precautions must be taken to avoid injury to the embryo. The following procedure gave uniformly good results: The point of a sharp dissecting needle was pushed into the membrane and the egg rotated so that the tip of the needle within the membrane could be held against the bottom of the dish at an acute angle. A second needle was then drawn across the under side of the first needle so as to make a slit in the membrane large enough for

the introduction of the point of the lower blade of the iridectomy scissors. By this means the membrane was readily removed, without exerting any pressure on the embryo. The naked embryos were kept overnight in sea water, during which time a few embryos which had been injured in the removal of the membrane died. The mortality was usually 4 to 5 per cent.

In the experiments recorded in this paper M/500 acetic acid was used in combination with varied concentrations of Na, K or Ca chloride. The mixture was made by adding 1 cc. of M/10 acetic acid to 50 cc. of the salt solution which gave a M/500 concentration of acetic acid without changing appreciably the concentration of the salt solution. Into each acid-salt mixture ten naked embryos were placed after having been previously rapidly transferred through two changes of distilled water to avoid carrying over an appreciable quantity of sea water. For controls other embryos were placed in distilled water and in M/500 acetic acid.

EXPERIMENTAL.

1. Standstill of the Heart in Relation to Other Manifestations of the Toxic Action of Acetic Acid.

Loeb's criterion of the toxic action of the acid was standstill of the heart. In a preliminary experiment we will consider the standstill of the heart in relation to other manifestations of the toxic action of the acid.

If naked embryos are immersed in M/500 acetic acid, no change from the normal is noted during about the first half hour. They remain motionless except for an occasional twitch of the tail. After the first half hour some of the embryos show evidence of irritation by more frequent and vigorous tail movements. Soon after this the tips of the tails of these embryos become white and opaque. This whitening and opacity gradually spreads until at the end of 3 hours the entire tail is involved. The heart remains normal until after about half of the tail has become white and opaque. The pericardium then starts to shrink and the heart is slowly retracted until it is almost all under the head. During this procedure the rhythm, conduction and contractility of the heart become severely and variously affected. Fig. 1 shows the time relation between the onset of tail injury and standstill of the heart. The primary manifestation of the toxic action of the acid therefore appears to be the injury to the tail of the embryo and this may be used as a criterion of the injurious action of the acid. Stoppage of the heart beat is always secondary to the injury of the

ectoderm of the tail. Embryos in acid show no disturbance of heart action so long as the surface of the embryo remains uninjured. Another constant feature of standstill of the heart as induced by acid is shrinkage of the pericardial cavity and retraction of the heart under the head. In addition, the standstill is complete and irreversible. It will be seen in a later experiment that the characteristics of heart stoppage of embryos immersed in KCl are very different from those in acid.

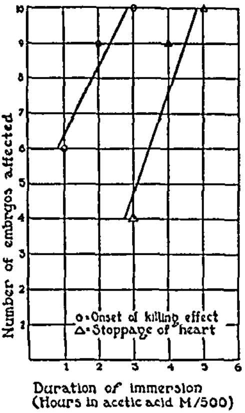

FIG. 1. Time relation between the onset of injury of the embryo and the stoppage of the heart in the acetic acid M/500.

2. *The Antagonism of Different Concentrations of NaCl to the Action of Acetic Acid.*

In this experiment naked embryos were immersed in NaCl-acetic acid mixtures. 1 cc. of M/10 acetic acid was added to 50 cc. of each different concentration of NaCl. This dilution gives M/500 acetic acid and the concentration of the salt solution is not appreciably changed. Table I shows that the higher concentration of the salt protected the naked embryos from the toxic action of the acid.

The results of this experiment are essentially the same as those reported by Loeb from his experiments with developing embryos enclosed within intact egg membranes. Apparently, therefore, the egg membrane plays no appreciable rôle in the antagonistic mechanism. In this experiment as in the preceding one, heart stoppage was always preceded by surface injury of the embryo.

TABLE I.

Antagonism of a Constant Concentration of Acetic Acid by Different Concentrations of Sodium Chloride.

Hrs. in solution	No. of embryos in which the heart continued to beat when immersed in 1 cc. of M/10 acetic acid in 50 cc. of:						Controls in distilled water
	Distilled water	M/64 sol NaCl	M/32 sol NaCl	M/16 sol NaCl	M/8 sol NaCl	M/4 sol NaCl	
3	10	10	10	10	10	10	10
4	8	2	8	10	10	10	10
5	3	0	0	10	10	10	10
6	0			10	10	10	10
7				9	10	10	10
8				4	10	10	10
9				1	10	10	10
11				0	10	10	10
19					8	10	10
26					7	10	10
44					4	7	10

3. *The Antagonism of Different Concentrations of $CaCl_2$ to the Action of Acetic Acid.*

According to Loeb different salts in the same concentration were not equally effective in antagonizing the toxic action of acetic acid, *e.g.*, he found $CaCl_2$ to be much more effective than NaCl. Table II shows the effectiveness of $CaCl_2$ in protecting naked embryos from the toxic action of acetic acid.

It can be seen that the higher concentrations of $CaCl_2$ behave like those of NaCl but that a M/16 solution of $CaCl_2$ is more effective than a M/4 solution of NaCl. Since this is the same effect reported by Loeb for developing embryos enclosed within intact membranes, the difference in the effectiveness of these salts cannot be due to the presence of the membrane.

4. *The Difference in the Way the Heart Stops in KCl and in Acetic Acid.*

If developing *Fundulus* eggs are immersed in either a solution of KCl or acetic acid, the heart stops beating. However, the mode of stoppage for each is specific. In a normal 5 to 8 day *Fundulus* embryo the heart shows a regular conduction of all beats from the sinus to the auricle and thence to the ventricle. If the embryo is placed in a KCl solution, this regularity is soon upset, so that only every other beat is carried through to the auricle and ventricle, and the ratio of beats in sinus-auricle-ventricle becomes 4–2–2. With continued immersion more of the beats in the auricle and ventricle are suppressed

TABLE II.

Antagonism of a Constant Concentration of Acetic Acid by Different Concentrations of Calcium Chloride.

Hrs. in solution	No. of embryos in which the heart continued to beat when immersed in 1 cc. M/10 acetic acid in 50 cc. of:					Controls in distilled water
	Distilled water	M/1024 $CaCl_2$	M/256 $CaCl_2$	M/64 $CaCl_2$	M/16 $CaCl_2$	
2	10	10	10	10	10	10
3	6	8	10	10	10	10
4	0	4	10	10	10	10
6		1	8	10	10	10
9		0	6	10	10	10
20			2	3	10	10
72			0	0	10	10

and the ratio becomes 4–2–1 or possibly 8–4–1. Finally the auricle and ventricle stop beating and the beating then persists only in the sinus. Nor does the entire sinus contract for the pulsation becomes restricted to a small bit of tissue about midway between the venous and auricular ends of the sinus. In the KCl solution there is no evidence of any surface injury to the embryo nor is there any shrinkage of the pericardial cavity and, if the embryo is returned to sea water, normal heart function becomes reestablished. In brief, stoppage of the heart by KCl may be identified by the manner in which it takes place, by absence of surface injury and pericardial shrinkage, by persistence of pulsation in sinus and by reestablishment of normal heart function on return of embryo to sea water. On the other hand

the action of acetic acid on the heart is preceded by surface injury of the embryo, the stoppage proceeds in no well defined steps, is accompanied by pericardial shrinkage, is complete and there is no reestablishment of the heart beat on returning the embryo to sea water.

5. *The Antagonism of Different Concentrations of KCl to the Action of Acetic Acid.*

Table III illustrates the antagonism of KCl to the surface-killing effect of the acid. The criterion of acid action is injury of the tail of the embryo as indicated by whitening and opacity. By the end of 3

TABLE III.

Antagonism of the Killing Effect of a Constant Concentration of Acetic Acid by Different Concentrations of Potassium Chloride.

Hrs. in solution	Embryos showing onset of injury in 1 cc. M/10 acetic acid in 50 cc. of:						Controls in distilled water
	Distilled water	M/64 KCl	M/32 KCl	M/16 KCl	M/8 KCl	M/4 KCl	
1	0	0	0	0	0	0	0
2	1	5	4	0	0	0	0
3	10	10	9	0	0	0	0
4			10	3	0	0	0
5-$\frac{1}{2}$				9	0	0	0
10				10	3	0	0

hours the hearts had stopped in all the solutions. In acetic acid plus M/64 KCl the mode of stoppage was characteristic of the acid effect, *viz.*, it was preceded by surface injury and was accompanied by shrinkage of the pericardial cavity, and was complete. In acetic acid plus M/4 KCl the mode of stoppage was characteristic of the KCl effect, *viz.*, the surface of the embryo remained uninjured, the stoppage was reached through definite steps, the pulsation persisted in the sinus and the pericardial cavity did not shrink. From this it follows that antagonism does not necessarily involve non-penetration of the salt for the M/4 KCl antagonized the surface killing effect of the acid but at the same time it penetrated and stopped the heart.

DISCUSSION.

Loeb (1, 2, 3) believed that several neutral salts including NaCl and $CaCl_2$ act similarly in that each can retard the rate of diffusion of acetic acid through the membrane of the developing *Fundulus* egg and so prevent or retard the toxic action of the acid on the embryo. If this is true then removal of the egg membrane should remove the protective action of the salt. In the three series of experiments described in this paper it was found that the protective action of the salt was as effective as ever after removal of the egg membrane. The effectiveness of this protection with NaCl was of the same order as that found by Loeb with the intact developing egg. With both intact eggs and naked embryos $CaCl_2$ was more strongly protective than NaCl. It is therefore unnecessary to attribute to the egg membrane any rôle in acid-salt antagonism since the results are quantitatively and qualitatively the same on naked embryos as on intact developing eggs of the same age. In a previous paper (5) we showed that, if intact eggs were immersed in either sea water or distilled water, the pH in the subchorionic space became that of the surrounding medium. The pH in the pericardial cavity, however, remained constant. It was suggested that it was not at the chorionic membrane that acid-salt antagonism took place. During the past season, Bodine has studied the action of Na, K and Ca chlorides on intact developing eggs and on embryos freed of their egg membranes. He states in a preliminary report (6) that the explanation of salt antagonism as advanced by Loeb needs certain corrections. In his experiments Loeb used the newly hatched fish as controls and found them to be very sensitive to acid-salt mixtures. However, such control material cannot be compared with the younger embryos. In the newly hatched fish the mouth and gills have broken through and the delicate surfaces of the gills are exposed to the action of the acid-salt mixtures whereas in the younger embryos the surface is still a continuous intact layer of ectoderm.

We must conclude, therefore, that the surface of the embryo is presumably the site of the acid-salt antagonism. It has been shown in this paper that the primary effect of the acetic acid was to injure the surface of the embryo. Stoppage of the heart was through second-

ary penetration of the acid. The presence of the salt prevented heart stoppage by preventing the surface injury. The antagonism at the surface of the embryo is not one in which the acid and salt each prevents the penetration of the other, which was Loeb's conception of the mechanism at the egg membrane. In the experiments in this paper on acetic acid-KCl antagonism, the higher concentrations of KCl antagonized the action of the acid on the surface of the embryo but at the same time the KCl penetrated and stopped the heart in the manner which is characteristic of KCl. That this stoppage was probably due to the penetration of KCl is further indicated by the fact that it is like that of the isolated terrapin heart immersed in KCl (4).

SUMMARY.

1. Developing *Fundulus* embryos react in much the same way to mixtures of acetic acid in salt solutions whether their membranes are removed or not. It is therefore not necessary to assume any specific rôle for the membrane.

2. The primary toxic effect of acetic acid is to kill the surface of the embryo; heart stoppage is due to penetration of the acid after the surface has been injured.

3. The salt (NaCl, KCl, $CaCl_2$) antagonizes the acetic acid by slowing or preventing the killing of the surface of the embryo.

4. If embryos are immersed in certain KCl-acetic acid mixtures, the surface killing effect of the acid is antagonized but at the same time the KCl penetrates and stops the heart in the manner which is characteristic of a KCl solution alone.

We take this opportunity of thanking Dr. Robert Chambers of Cornell University Medical College for valuable advice given in the preparation of this paper.

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POTENTIALLY UNLIMITED MULTIPLICATION OF YEAST WITH CONSTANT ENVIRONMENT, AND THE LIMITING OF GROWTH BY CHANGING ENVIRONMENT.

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I.

A small amount of yeast seeded into a given volume of a suitable culture medium grows at a constant rate for a short time, after which the growth rate gradually decreases until the yeast just maintains itself at an equilibrium crop. The experiments reported in this paper show that the constant rate of growth can be maintained for as long as the environment can be maintained effectively constant. The part of the usual sigmoid growth curve of the yeast population after the inflexion point is merely a measure of the retarding effect of the accumulation of waste products. If the retarding influence is avoided the growth curve for a population of yeast is an exponential curve. Consequently, an attempt to analyze directly the nature of the S-shaped curve to determine the basic nature of the growth process can not be successful. This is why the method for such an analysis proposed by Robertson (1923) has not been profitable, even with the most general conditions.[1] The growth in diameter of mold colonies gives a sigmoid curve (*cf.* data in Fawcett, 1921), but as Crozier (1926) has indicated this curve is complicated by changes in the substratum not unlike those to be discussed in the case of yeast.

The experiments were made partly in the Department of Zoology of the University of Oregon, and partly at the Laboratory of General Physiology of Harvard University, and will be referred to as the Oregon and the Cambridge experiments respectively. The origin of the strain of yeast used in the Oregon experiments,

[1] Richards. O. W., The growth of the yeast *S. cerevisiæ*. I. The growth curve, its mathematical analysis and the effect of temperature of the yeast growth, *Ann. Bot.*, 1928, clxv, 271.

the culture technique, and the method used for determining the growth have been described.[1]

The Cambridge experiments were made with another strain, No. 2335: *S. cerevisiæ*, Hansen, furnished through the kindness of Professor F. W. Tanner of the University of Illinois. Its growth is the same as that of the strain used at Oregon, except that the equilibrium crop is numerically slightly different.[2] Except as specifically stated, the technique used in the Cambridge and in the Oregon experiments is the same.

II.

The effect of crowding was mentioned, but not tested, by Carlson (1913). Clark (1922) found that crowding did not lessen the growth when air and fresh medium were forced through the culture for 5 hours. I have found that rocking the cultures of yeast slightly increased the yield but did not change the nature of

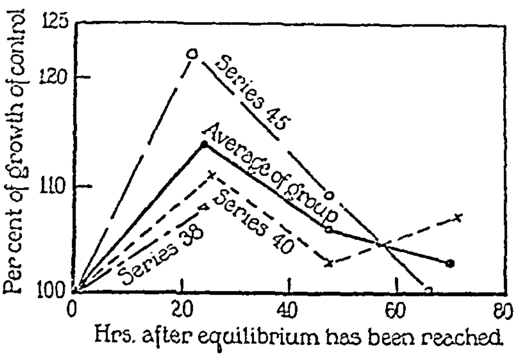

FIG. 1. The effect of reseeding a culture that has reached an equilibrium of growth.

the growth curve. The increased yield is not sufficient to justify the use of a rocking device.

Other experiments[1] have shown that a decrease of food in the medium is not a causal factor in the decrease of the growth rate. When the growth rate is diminished by a lack of food it is usually due to the exhaustion of an essential substance rather than to a general decrease of all of the nutrient substances (Slator, 1921). This is supported by the fact that diffusion seems to be adequate to furnish nutrient material to the individual cells (Slator and Sand, 1910).

Two series of experiments were made by growing the same seeding of yeast in the same volume of culture medium in both petri dishes and in test-tubes. The layer of medium in the petri dishes was about 1 mm. thick, so that the surface was

[2] The significance of these differences is discussed in the previous paper,[1] together with the method used in averaging the results of the different experiments.

proportionally large as compared with the small surface and the greater depth of the test-tube cultures. The amount of growth in each case was so nearly the same that the probable errors of the determinations overlapped throughout the time of the entire cycle. This shows that oxygen is not a complicating factor in the present study.

If the figures for any given series are studied the equilibrium crop is seen to be reached actually by oscillations. The maximum variations amount to less than 10 per cent for the test-tube cultures used in the present study. These oscillations vary from series to series and average out when several series are combined which gives the smooth line on Fig. 4*A*. This indicates that the oscillations are caused *by changes in the environment rather than due to primary causes*, as Ludwig (1926) seems to infer from the greater magnitude of the oscillations (about 30 per cent) found in his four 1-liter mass cultures.

TABLE I.

Size of Cells and Freezing Point Determinations at Equilibrium Conditions.

	Average cell size*	Δ	Per cent size	Per cent Δ
Control	91.5	0.434	100	100
Replaced	130.0	0.514	142	118
Doubled	127.8	0.577	139	132

* The difference of average size (arbitrary units) in these experiments and the previous ones is merely due to different enlargements of the photographs, as they are from the same strain of yeast and under the same condition of culture.

If a culture that has reached equilibrium growth at about 100 hours after seeding be reseeded with the usual seeding of cells, 40 to 48 hours old, we find a slight but definite increase in the number of cells present. The new growth soon ceases and the result is a new equilibrium at a higher level (Fig. 1), which seems to show that there are present in the medium products either toxic to the new buds produced or actually preventing budding.

Withdrawing half of the culture medium seems to have little effect on the cells. The average size of the cells is 65.1, as compared to 63.7 area units[3] of the controls, a difference just a little less than the error of measurement and hence insignificant. If the medium is pipetted off from the cells, and an equal volume of fresh, sterile medium added, we find an increase in the total volume of the yeast present of

[3] These data were obtained by measuring the tracings of projected photographs of the yeast cells with a planimeter, and they are expressed in arbitrary *area* units. A more complete discussion of the size changes of the cells and of the method will be published in a separate paper.

about 100 per cent, and about 40 per cent increase in the number of cells present. The average cell size is 74.1 units or 116 per cent of that of the controls, at 70 hours after making the change.

Adding an equal volume of fresh, sterile medium to the amount already present increases the volume of yeast by about 80 per cent and the number of cells by about 30 per cent in the same interval of time. The average cell size in this group is 71.0 units, or 111 per cent of the average size of the control yeast.

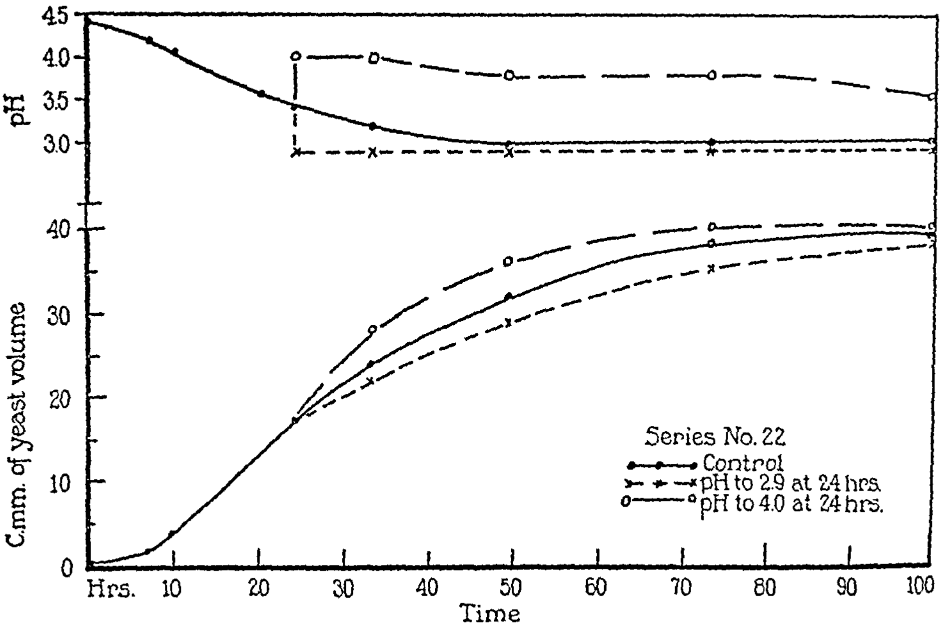

FIG. 2. The effect of changing the acidity of the culture medium on the growth of yeast.

These results support the view that the inhibition of the growth of the population is due to toxic waste products present in the medium. Doubling the amount of the solution merely halves the amount of toxic substances present, while replacing the medium greatly reduces their concentration. The increased yield was considerably greater with the latter condition than with the former. The changes of osmotic pressure in such experiments, proportional to the depressions of the freezing point, are opposite to the changes in the size of the yeast cells, as shown in Table I. The size changes are therefore not simply the result of osmotic pressure changes.

III.

The curve showing the change of acidity of the culture medium as the population grows is similar to the growth curve inverted (Fig. 2).

The acidities were determined colorimetrically by means of a drop method. The values were found to be slightly more accurate than 0.1 pH when checked with the aid of a hydrogen electrode.[4]

The effect of the acidity of the medium may be tested by dividing a number of tubes into groups, at about 24 hours after seeding, and changing the acidity in some while retaining the rest for controls. Some of the tubes were brought, with a few drops of N/10 HCl, to a pH below the value that they were expected to reach. The other group of tubes was partially neutralized with $Ca(OH)_2$ to about the acidity that they had at the time of greatest velocity of growth. Fig. 2 shows that acidity reduced the yield of yeast and that partially neutralizing the medium increases the crop.

Continued partial neutralization increases the crop and the growth curve is observed to be more symmetrical. However, the equilibrium conditions are merely delayed and not prevented, so the acidic changes are not solely responsible for bringing the growth to an equilibrium. The anion of the acid added may be deleterious to the yeast as well as the increase in the hydrogen ion concentration. The Ca is probably not toxic as the amount added does not bring the total concentration of the Ca above the optimum concentration previously found (Richards, 1925). The optimum Ca concentration would cause less increase than that noted in these experiments.

The effects of changing the acidity of the medium are summarized in Table II. These effects are not due to the dilution of the medium, because a similar simple dilution has no effect on the growth. When NaOH is used for partial neutralization it is strongly toxic. A greater yield of yeast may be obtained by using K_2HPO_4 instead of KH_2PO_4 in the medium, but the shape of the growth curve is hardly altered. Further buffering makes the medium toxic.

These effects seem to be general, as Yeast 2342 of the American Type Culture Collection responds in the same way and the results were duplicated at Cambridge with a third strain. All of these strains are "bottom" yeasts and give similarly shaped growth curves (with numerically different levels of equilibrium).

[4] I am indebted to Professor J. B. Conant for having these determinations with the hydrogen electrode made in his laboratory.

The maximum velocity of the change in acidity of the medium occurs *after* the maximum velocity of the increase in the number of cells. The Cambridge experiments gave maxima of 30 and 50 hours for the growth rate and the rate of change of acidity respectively. This demonstrates that the increase in acidity is an accompanying rather than the primary cause of the decline of the rate of growth of the yeast population. This is strengthened by the fact that the difference between the growth at different acidities is not numerically proportional to either the pH or the C_H. The acidity is due to non-volatile organic acids, because boiling a tube that has reached a pH of 3.0 only changes it to pH 3.2. Saturating the medium with CO_2 at 24°C. brings the acidity only to 3.3. The nature of the acid present will be considered again in Section IV.

TABLE II.

The Effect of Changing the Medium on the Growth of Yeast (Oregon Experiments).

Hrs. of growth	Percentages of the central group		
	Kept at pH 4.0	To pH 4.0 at 24 hrs.	To pH 3.0 at 24 hrs.
35	108	111	91
48	116	110	86
72	107	104	90
100	105	102	90

When yeast is grown in sealed tubes CO_2 has been shown to be a limiting factor for growth (Slator, 1921). Many investigators have observed that CO_2 lessens the growth of yeast, but few quantitative investigations have been made. My observations do not support the suggestion made by Pearsall (1925) that optimum cell growth occurs at or near the "isoelectric point" for the cells, or of the tissues of higher plants. Pearsall quotes the isoelectric point for yeast suspensions, as determined by cataphoresis, as pH 3.1 to 3.3. Certainly the growth of yeast is slight at such acidities, and they are reached long after most rapid growth has ceased. However, the isoelectric point for cell suspensions is probably very different from that of the cell contents at the place where the bud is being formed. Until we can measure the "isoelectric point" of the cell constituents that are being modified into a bud, such a theory must be speculative.

IV.

Most investigations on the effect of alcohol on yeast have concerned the fermentation industry and yield little of value to the present study. The earlier data are summarized by Euler and Linder (1915).[5] Clark (1922) found that the growth of yeast was logarithmic for about 15 hours, when it began to decrease. Experiments showed that alcohol concentrations in excess of 1.75 per cent slowed growth. Clark does not give his method for determining the amount of alcohol further than "by distillation." He concludes that the retarding effect of alcohol is due to a lowered rate of reproduction of each cell.

A few experiments with the strain of yeast used in Oregon confirmed the observations of Clark. The chief difficulty of such experiments is the accurate determination of the amount of alcohol present in the 10 cc. of medium used in each tube. The most satisfactory method was a modification of the Nicloux method used by Miles (1922). It gave accurate checks when as little as 1 mg. of alcohol was present per cc. Alcohol production detectable by this method does not take place until after 24 hours after seeding. The alcohol then increases, reaching a maximum at 40 hours, and then decreases until an equilibrium concentration is reached (Fig. 4*D*).

Comparison of the individual curves indicates that the rate of growth of the yeast population immediately declines after the alcohol concentration reaches 1 mg. per cc. of medium. This is equally true when the acidity is maintained constant, which suggests that it may be a real threshold concentration. That the acidity effect in slowing the growth of yeast is secondary to the alcohol effect is attested by the maximum acidity increase occurring 10 hours later than the maximum alcohol production. Pyruvic acid, which is an accompaniment of the alcoholic fermentation, first appears at about 44 hours, or about 5 hours before the acid production becomes maximal.

These relations were tested as follows: the tubes were divided into three lots and one lot was kept for a control, the other two receiving sufficient alcohol to make the concentration 12 mg. per cc. of medium, which is more than the amount usually produced. One lot was maintained at a pH greater than 3.9, by frequent neutralization, and the

[5] See Euler and Linder (1915), p. 283.

other lot was unchanged (Fig. 3). In both cases the addition of alcohol reduced the growth of the yeast. Holding the acidity relatively constant seems to stabilize the growth curve. A decreased amount of alcohol is associated with this partial neutralization and there is also

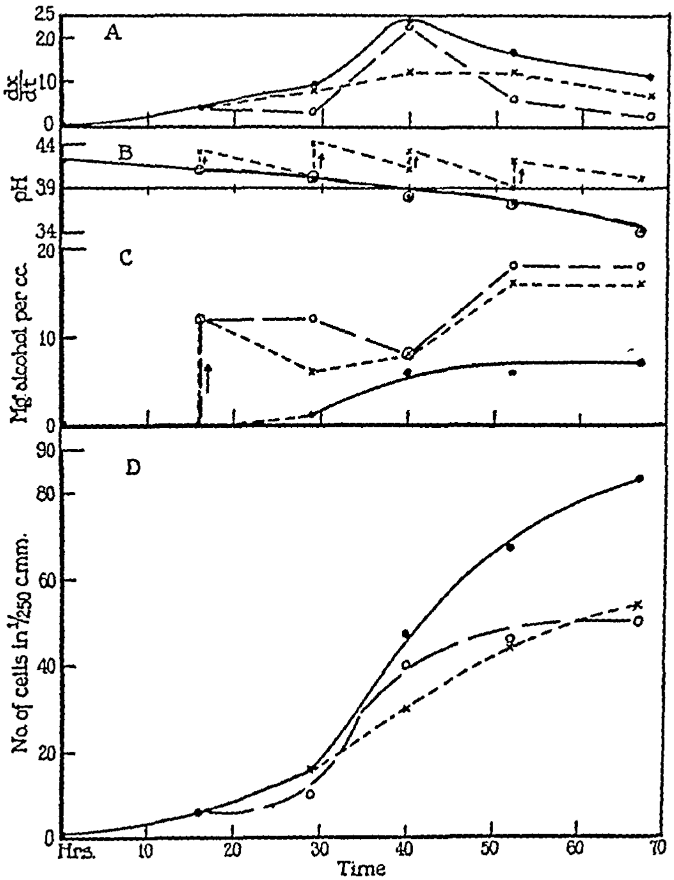

FIG. 3. The effect of alcohol on the growth of yeast. *A*. Velocity. *B*. Acidity *C*. Alcohol production. *D*. Growth curves. ● = Control group; ○, group + alcohol; x, group + alcohol kept at pH > 3.9.

a delay of the alcohol effect on cell proliferation. Adding alcohol only at first depressed, then accelerated, and finally depressed the growth more rapidly than in the control. The growth yield with the alcohol added was 59 per cent of that of the control, and with the alcohol added and the acidity controlled the yield was 65 per cent of

that of the control. The alcohol production was twice that of the control in the group maintained at approximately constant acidity, and slightly greater in the group not kept at constant acidity. The addition of the alcohol did not change the rate of acid production. This again suggests that the alcohol effect is the primary cause of the decrease in the rate of growth of the yeast population.

This strain of *S. cerevisiæ* is more sensitive to alcohol than that used by Clark (1922). The effect of alcohol may be determined by finding the percentage of cells that are actually budding at the time of greatest growth rate and at the time the population is just maintaining itself at a certain equilibrium. At 30 hours, time of maximum velocity of growth, and at 116 hours, culture at equilibrium, these percentages are 22 and 19, respectively, for the average of two series of experiments. As will be shown in a later paper, the distribution of the sizes of cells indicates that it is the larger buds that are selectively affected by the increased toxic products in the environment, rather than the division rate as suggested by Clark.

My experiments suggest that it is the increase of alcohol rather than the exhaustion of sugar that causes the decrease of the rate of the growth of yeast in the aerated, ammonia-molasses medium used by Balls and Brown (1925). They report that the sugar is used up by 8 hours of growth. Calculation shows that the velocity of the yeast growth begins to decrease steadily at about 5 hours. At 5 hours the alcohol content of the medium is from 1.6 to 5 mg. per cc., which is near the threshold found in the present experiments. Further, Balls and Brown failed to obtain any distinct increase in the growth of their yeast by renewing the sugar after it had been exhausted. Both of these facts suggest an alcohol effect on the growth rate instead of the exhaustion of the sugar. The growth curves of their experiments and of mine are very similar, which indicates that aeration increases the yield of yeast but does not change the sequence of events in the growth of the yeast population.

V.

One cannot run fresh medium over the cells without losses, if caking on a filter is to be avoided. The best method for getting rid of the alcohol seemed to be to very carefully pipette the medium off down to

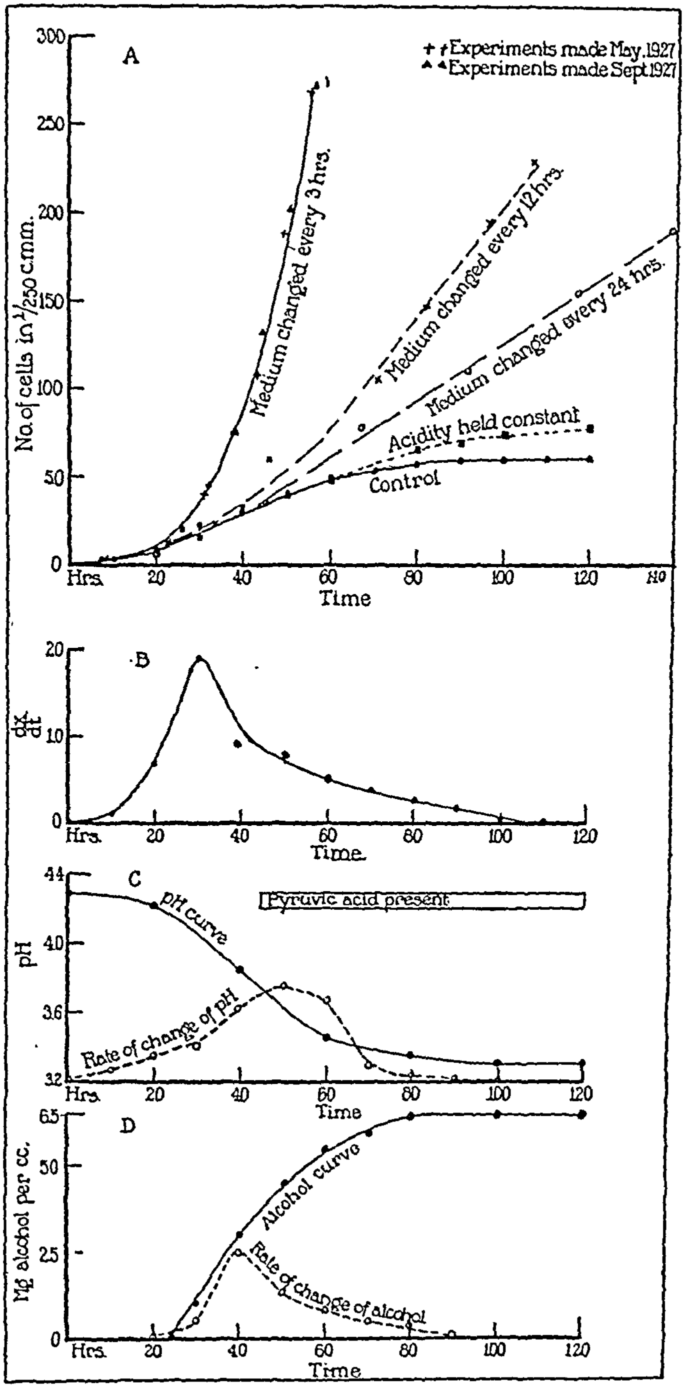

FIG. 4. *A*. Growth curves. *B*. Velocity curve of the control. *C*. The changes in acidity of the medium of the control. *D*. The alcohol production of the control.

as close to the cells as possible without actually removing the cells. This left about 0.3 to 0.5 cc. of medium, but the concentration of waste products contained in it would be greatly reduced when the original volume (10 cc.) was restored with fresh, sterile medium. The amount of alcohol left could be kept to an amount below the threshold value by frequently changing the medium.

Changing the medium at intervals of 24 hours, Figs. 4*A* and 5, shows a yield about three times as great as that obtained by the control group. The growth after 60 hours is practically linear to the time

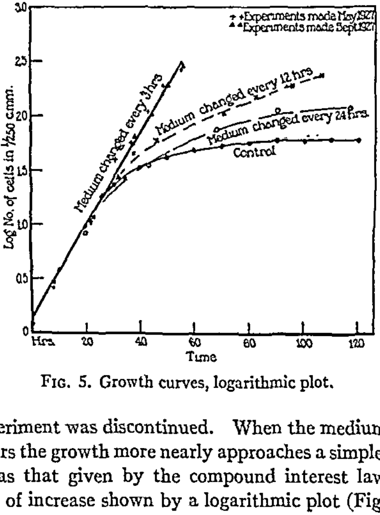

FIG. 5. Growth curves, logarithmic plot.

that the experiment was discontinued. When the medium is changed every 12 hours the growth more nearly approaches a simple logarithmic curve such as that given by the compound interest law (Fig. 4*A*), and the rate of increase shown by a logarithmic plot (Fig. 5) remains constant for a longer time.

Inspection of the times of departure from the control growth curve, or of the slopes of the growth curves after the departure from the linear segment, indicates that changing the medium every 4 hours should entirely prevent the inhibiting effect of the toxic products.

The medium was consequently changed every 3 hours. This gave

a relatively enormous growth that had to be discontinued at abou hours, as the number of cells became so great as to prevent chan the medium without losing some of them. A further increas numbers would require dilution before they could be counted. spection of the logarithmic plot in Fig. 5 shows that the curv linear within the experimental error. The smooth line was dr through the points representing experiments made in May. experiment was repeated in September and the points of the la test are plotted on the previous graph. The agreement of the de minations indicates the orderliness and regularity of the growth of yeast under these conditions.

Multiplication under the condition of an effectively constant vironment as obtained by changing the culture medium every 3 h is *potentially unlimited.* The increase in the number of cells per of time is in proportion to the number of cells present, and foll the compound interest law. This form of growth leaves no room the assumption of any autocatalyst for multiplication, because rate of increase is constant.

Slator (1913) has obtained logarithmic growth up to a concen tion of about 10 million cells per cc. My yield is about 70 mill cells per cc. This may be relatively a greater crop, as I have use synthetic medium while Slator used a wort medium. Clark (19 maintained logarithmic growth for almost 24 hours, or for a li more than a third of the time in the present experiment. By us larger dishes and improved technique for changing the medium constant rate of growth could be greatly prolonged.

Consequently, when the food is adequate, the sigmoid part the growth curve after the point of inflexion is merely a measure the inhibiting effect of the toxic waste products of the cells, excre into the environment. This suggests that the results of experime regarding the relations of yeasts to vitamines, for example, can o be compared when the growth is maintained in a constant envir ment, and probably accounts for some of the current controversy this field (*cf.* Tanner, 1925). It lends further weight to the opini expressed by some investigators that probably the conditions multiplication are more significant in influencing the rate and nat of growth than any "bios" (Tanner *et al.*, 1926).

It is quite possible that the growth of populations of other unicellular organisms is similar to that found for yeast. The fact that yeast grows by budding does not influence the rate of growth under constant conditions. The relations between the individual cell and those of the population will be considered in more detail, together with the changes in the sizes of the cells during growth, in a forthcoming paper. The effect of variable and constant environment on the growth of populations of yeast suggests that analogies between yeast and human populations, such as have been made by Pearl (1925), should only be made with the greatest of caution.

SUMMARY.

1. The decrease in the rate of growth of a population of yeast cells, which results in the maintenance of an equilibrium crop level, is shown to be due to substances excreted into the culture medium by the growing cells. These toxic substances tend to destroy the young buds, because the percentage of budding cells is about the same at the time of most rapid growth and at the time of the growth equilibrium.

2. Alcohol is the product which primarily causes the decline of the growth rate. For the strain of yeast used, under the particular conditions of these experiments, a concentration of alcohol of about 1 mg. per cc. is associated with the beginning of the decrease of the growth rate.

3. The increasing acidity of the medium, due to CO_2, pyruvic acid, and other organic acids, is also a retarding influence. It is a secondary factor, however, as the greatest increase of the acidity of the medium occurs after pyruvic acid, probably a by-product of alcoholic fermentation, appears.

4. When the medium is maintained effectively constant, by preventing the accumulation of these toxic products, the yeast grows at a *constant* rate and the yeast growth is *potentially unlimited*. The limit of growth found in actual experiments is due only to the size of the test-tubes and to the relative efficiency of the method used in keeping the medium effectively constant. The necessity of maintaining a constant rate of growth in studies on the relations of yeasts to vitamines and other products is stressed.

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MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

VI. Calcium Ions in Living Protoplasm.

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In studying the chemistry of protoplasm it is essential to permit as little variation from the normal state of the cell as possible. The mass of data accumulated on the analysis of dead tissues reveals little information concerning its actual state during life. A striking example is afforded in studying protein precipitation in living cells and in extracts of dead cells. An alcoholic solution of picric acid, for example will precipitate protein from its aqueous extracts of dead cells within wide ranges of pH, whereas one may inject such a picric acid solution, in comparatively large quantities, into the living ameba with practically no toxic effect (1). Another example of the difference in the chemistry of living and dead or injured cells is seen in the study of the hydrogen ion concentration (2, 3), in which it has been shown that the pH of the normal cytoplasm of echinoderm ova is about 6.8, while the injured cytoplasm has a pH of about 5.4, and the dead mass assumes the pH of its medium.

The application of chemical tests to the living cell is subject to many limitations. First, the reagent must react at the pH of the cytoplasm. Secondly, color tests are to be preferred because of the difficulty of watching precipitations in the cell with ordinary illumination. Third, the reagent should be relatively non-toxic. Fourth, the reagent must be very sensitive.

The recently reported values for the pH of the ameba lie between 6.9 (4) and 7.6 (5, 6). This restricts the reagents that can be used in the ameba to those that are most efficient in a medium close to neutrality.

It was found that alizarin sulfonate will precipitate calcium ions

quantitatively while conforming to the above conditions. The resulting compound, calcium alizarinate, can be seen under the microscope in the form of purplish red crystals. When the pH is close to neutrality and there is an excess of calcium one would expect no appreciable interference from the possible presence of magnesium in the cell. The toxicity of this reagent is discussed in the experimental section below.

Alizarin has been used (7) for the study of bone growth, by feeding it in the form of madder root to experimental animals. The justification for this practice seems to be that alizarin combines with calcium in the bone to form red colored insoluble calcium alizarinate. Alizarin has also been used recently to determined blood calcium (8).

EXPERIMENTAL.

1. Injection of Alizarin.

By means of the micrurgical technique (9) varying quantities of a saturated aqueous solution of sodium alizarin sulfonate were injected into *Amœba dubia* and *Amœba proteus*.

The injection of a moderate quantity (1/4 the volume of the ameba) of a saturated aqueous solution of this reagent (reddish brown in color) causes a temporary cessation of movement. The ameba rounds up and the larger crystals and granules may settle to the bottom. A close examination of the cytoplasm shows fine purplish red granules scattered throughout the cell, and the hyaline cytoplasm itself is diffusely colored pale red.

If the ameba attempts to put forth a pseudopod as evidenced by a slight lifting of the membrane a shower of these purplish red granules are seen to appear in this area and the pseudopod formation is immediately stopped. During recovery the diffuse red color gradually disappears but there is an appreciable increase in the number of the purplish red granules.

The return to normal activity is gradual, generally lasting over a period of 2 to 3 hours. A peculiar phase in the process of recovery is the elevation of the plasmalemma and the appearance of a prominent hyaline zone between it and the condensing granuloplasm. The surface boundary of the granuloplasm soon breaks down and a portion

of the granuloplasm starts to flow into the hyaline zone, slo first, then with increasing rapidity. The movement then sto granuloplasm again separates itself from the hyaline zone, a process is repeated. Reznikoff and Chambers (10) have call phenomenon a pseudomembrane formation.

If an ameba is killed during the injections or is torn by the needles in a medium containing alizarin, the large crystals no present in the ameba and some of the coagulum which is pr upon death will also take on the purplish red color characteri calcium alizarinate.

The nucleus may also be affected by the injection. Wher affected it loses its normal granular appearance and appear hyaline body with a pinkish brown stain. The ameba may e such a nucleus and recover to the extent of actively moving fo time.

2. Injection of Calcium Following the Injection of Alizari

The quiescence which is induced after an injection of alizari be due to a removal of calcium of the protoplasm from the sp action. The idea suggested itself that a subsequent injectio solution of calcium chloride should aid in the recovery of the am

It has been shown (11) that the injection of calcium chlorid tions in concentrations stronger than M/208 causes an injury form of a local coagulation (11). Therefore the concentration were limited to M/208 and less.

When an ameba which has previously been injected with aliz injected with an M/208 calcium chloride solution, active flowing ments appear almost immediately which subside in a very short Another injection of the calcium solution into the same ameba h same effect. Because of the resulting trauma no more tha successive injections were tried on any one ameba. The time u required for complete recovery after an alizarin injection is sho from about 2 to 3 hours to $\frac{1}{2}$ to 1 hour.

3. Injection of Other Calcium-Precipitating Anions.

The similarity of the effects of the injection of the phos carbonates, and sulfates as observed by Reznikoff and Chambe

to those observed on the injection of alizarin is very close. This is of interest, since these three anions also form insoluble salts with calcium. To further investigate this uniformity of action of the calcium precipitants, injections were made of the salts of two other organic anions whose calcium salts have relatively low solubility products, *viz.*, tartrate and oxalate.[1]

It was found that the effect of these injections was practically the same as that of alizarin. The ameba could recover from a moderate injection of M/8 solution of sodium potassium tartrate or of M/18 solution of sodium oxalate after going through the stages of quiescence, rounding, and pseudomembrane formation. Concentrations as low as M/128 of sodium potassium tartrate and M/620 of sodium oxalate still called forth a pseudomembrane reaction. If the nucleus became hyaline, the ameba would not recover unless it extruded the hyalinized nucleus.

DISCUSSION AND SUMMARY.

The quiescence, rounding, sinking of the granules, and paling of the nucleus are similar to the effects seen after the injection of potassium and sodium chloride (11). Since the sodium salts of the anions were used, it might be inferred that the sodium is the active agent in the injected solutions. This is not entirely the case, however, for the effective concentrations of NaCl required are many times greater than those required in the case of the sodium salts of the calcium-precipitating anions. The fact that practically the same effects can be obtained in both cases leads one to suspect that there is a relation between the results of an increase in sodium ions and a decrease in calcium ions. It has been shown that a M/416 $CaCl_2$ solution will antagonize a M/1 NaCl solution and even a more concentrated solution of KCl inside the ameba (12). Therefore the reduction in amount of calcium may leave a comparatively high concentration of unantagonized sodium and potassium.

The fine, purplish red granules resulting from the injection of the alizarin are, no doubt, the insoluble calcium alizarinate. Recovery of an ameba from such an injection may be explained by the postulate

[1] I take this opportunity to thank Dr. Ruth B. Howland for her kind help with these injections.

that the free calcium ions in the living ameba are in equilibrium with a reserve supply of unionized calcium. The equilibrium is upset when the free calcium is removed by precipitation or by other means, and the system may possibly react in such a way as to counteract the effect of the change imposed. By mobilization of the calcium from a reserve supply the ameba can therefore gradually resume its normal activity. The time required for the recovery depends on the amount of alizarin injected. The diffuse red color which is seen immediately following the injection of alizarin probably represents that extra amount of dye which was not used in precipitating the immediately available calcium. Then, as the calcium is being liberated from the reserve, it is taken up by this surplus alizarin, resulting in a gradual loss of the diffuse coloration and an increase in the number of purplish

TABLE I.

Salt injected	Toxic concentration of injection of 1/4 volume of ameba	Water effect	Solubility product of corresponding calcium salt
Na_2SO_4	M/2	M/64	6×10^{-5}
NaK Tartrate	M/8	M/250	7.7×10^{-7}
Na_2HPO_4	M/16	M/1280	5.4×10^{-7} $Ca_3(PO_4)_2$
Na_2Oxalate	M/16 M/18	M/1860	1.7×10^{-9}
$NaHCO_3$	M/32	M/256	1×10^{-8}

red calcium alizarinate granules. Only when all of the injected dye has been precipitated can the mobilized calcium be used to carry on the normal physiological processes of the organism.

The need of calcium to effect ameboid movement has been shown by Pantin (13) in a series of immersion experiments. This fact is quite suggestive, because the first effect of the injection of any of the calcium precipitants is absolute quiescence. Furthermore, there is no return to normal movement until the calcium apparently becomes available to the protoplasm.

In support of the conception of a reserve supply of calcium is the presence of the large crystals which give a positive reaction with alizarin for calcium on the death of the ameba. Schewiakoff (14), from crystallographic studies, claims that they are calcium phosphate.

The effect of the injection of the calcium-precipitating anions on the calcium of the protoplasm may be shown in another way. In determining the relative toxicity of these salts an arbitrarily standardized injection, about one-fourth of the volume of an ameba, was used. This was introduced because of the necessity to avoid effects due to variable amounts of the solvent, *viz.*, water. Thus the water effect was kept constant, and the variations in actual amount of salt injected were obtained by using a graded series of concentrations.

Arranging the sodium salts of these anions in order of increasing toxicity in one column, and the *in vitro* solubility products of the corresponding calcium salts in another column, it is seen that as the toxicity increases, the solubility product decreases (Table I). This fact strongly suggests that the toxicity depends on the ability of the salt to remove calcium ions from the protoplasm. The apparent deviation of the carbonate from the rule can be explained by the specific effect of CO_2 (10) which is always present from the hydrolysis of the carbonate.

CONCLUSIONS.

1. The injection of alizarin sulfonate gives a color test which demonstrates an appreciable amount of free calcium ions in the living ameba.
2. The toxicity of the anions: phosphate, sulfate, tartrate, and oxalate is related in some measure to the solubility product of the corresponding calcium salt. The carbonate does not fall in line.
3. The ameba has a calcium reserve which serves as a mechanism of recovering from the effects of sublethal doses of these anions and of the alizarin sulfonate.
4. The post mortem color reaction with alizarin sulfonate gives evidence of the presence of calcium in the large crystals in the ameba.
5. The behavior of the ameba is practically the same when the intracellular calcium ion concentration is diminished or when the sodium or potassium ion concentration is increased.

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TEMPERATURE CHARACTERISTICS FOR PULSATION FREQUENCY IN GONIONEMUS.

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I.

The analysis of vital activities in terms of specific governing processes through their quantitative control by temperature (*cf.* Crozier, 1924, 1924–25, *b*) requires as an essential preliminary the collection of adequately determined values of the temperature constants or *temperature characteristics* (Crozier, 1924–25, *a*), μ of the Arrhenius formula. As obtained from a variety of experiments with the most varied biological phenomena the magnitudes of this constant are found to be grouped in a manner indicating specific significance (Crozier and Stier, 1926–27, *b*), and this is true also where several values of μ are found associated with the same kind of phenomenon under different circumstances in the same kind of organism (*cf.* Crozier and Stier, 1925–26, *a*, *b;* 1926–27, *a*, *c*). The fact that activities of the same general kind in organisms not too distantly related appear to provide concordant magnitudes of μ independently encourages an objective classification of the dynamics of these activities (Crozier, 1924–25, *a;* Crozier and Stier, 1924–25, *a*, *c;* 1927–28; Glaser, 1925–26, *a*, *b;* Crozier and Federighi, 1924–25, *a*, *b;* Fries, 1926–27). The disturbances which may be introduced into the determination of temperature characteristics by the occurrence of critical temperatures at which the quantitative relationship between temperature and rate or frequency is altered, and by the precise sort of alternation encountered, have been discussed in various previous publications (Crozier, 1924–25, *b;* Crozier and Stier, 1924–25, *b;* 1926–27, *a*, *c*). For certain instances in which there is measured the dependence of frequency of action upon tempera-

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ture, the value of μ at first sight has seemed non-specific. One such case has been recorded by Murray (1925–26) in the beating of explants of embryonic chick heart. In explanation of it the suggestion has been made (Crozier and Stier, 1926–27, *c*) that where more than one local pace maker is functioning, but with differing intrinsic values of μ, there should result distributions of the observations exactly of the general types found. Certain deductions from this interpretation, or connected with it, have been tested (Crozier and Stier, 1926–27, *c*). It was pointed out (*loc. cit.*) that the effect invoked might under certain circumstances be duplicated experimentally with the hearts of tunicates or with the pulsating swimming bell of medusæ. The work of this paper has been devoted to the examination of the relations between temperature and frequency of contraction in *Gonionemus.*

A number of papers have been published in which this question has been discussed, especially for scyphomedusæ (*cf.* Mayer, 1914; Cary, 1917; Schaefer, 1921). It must be said, however, that in no case are the data sufficiently numerous to permit more than suggestive deductions as to the magnitudes of μ which may characterize pulsation frequency, and as to the occurrence of critical temperatures. There are required very numerous readings, upon each of a good number of individuals, in order that the latitude of variation at constant temperature may be adequately dealt with (*cf.* Crozier and Federighi, 1924–25, *a;* 1925; Crozier and Stier, 1924–25, *a*, etc.; Glaser, 1925–26, *a*). This is particularly important when the frequency as measured is high, and the possibility of observational error therefore increased. Before employing these medusæ for the experiments ultimately in view it is necessary to determine if the frequency adheres to the Arrhenius formula, and if there may not be differences among "normal" individuals, as well as to test the effects of certain operations upon the marginal sensory ring. The earlier work upon medusæ has indicated that any of the many marginal sensory organs may be brought to serve as pace maker, the fastest beating region of the margin determining the pulsation of the whole (Loeb, 1900; Mayer, 1906, 1910; Bozler, 1926). The question first to be decided is the degree of uniformity exhibited in the relationship to temperature when there is a possibility that not all the marginal organs are metabolically in exactly comparable states, so that (in terms of the

general theory already discussed) they might not exhibit the same temperature constants.

II.

The material used in these experiments was collected in the Eel Pond at the Marine Biological Laboratory, Woods Hole, during summer months. The individuals varied between 5 and 25 mm. in diameter. In the different experiments animals of all available sizes were used. The activity of these is in general independent of size. There are, however, differences in the number of pulsations per unit time; but this does not affect the way in which the frequency of pulsation changes regularly with the change of temperature. Altogether 76 animals were used during the experiments, 44 during July and August and 32 after a short interval in September, for a reason which is explained later. 5130 stop-watch readings were taken, the greater number for 10 contractions each, a smaller for less than 10, in cases where the animals did not beat regularly enough to get 10 pulsations between pauses. In the figures there are plotted 662 points, representing averages.

The animals were placed in a glass beaker submerged in a large glass vessel in which the temperature could be controlled by adding ice or hot water and kept constant within 0.2°C. during an interval of 10 to 20 minutes. Freshly collected animals are rather active and are in motion most of the time. If however they are kept in the aquarium for several days, beating occurs only seldom. In these cases the animals were touched with a thin glass rod and thus caused to swim actively for awhile. During rainy and cloudy days the activity is only partly as good as on clear days. Animals collected during bad, rainy weather are almost impossible to use for these experiments because the pulsations are not sufficiently regular.

The individuals used are to be divided in six classes, according to the operations made. The first class are normal animals, without any operation or known interference. The second group are animals where the nerve ring was cut twice, on opposite sides of the margin of the bell. The third class are animals where the nerve ring was entirely removed. Class 4 contains animals with one cut through the nerve ring; Class 5, animals where the nerve ring was cut four times; class

6, animals cut in half. The results obtained during July and August were plotted in terms of the Arrhenius equation. In the beginning of September the experiments were repeated. Each of the different groups of animals was tested again. The observations entirely paralleled the results obtained before.

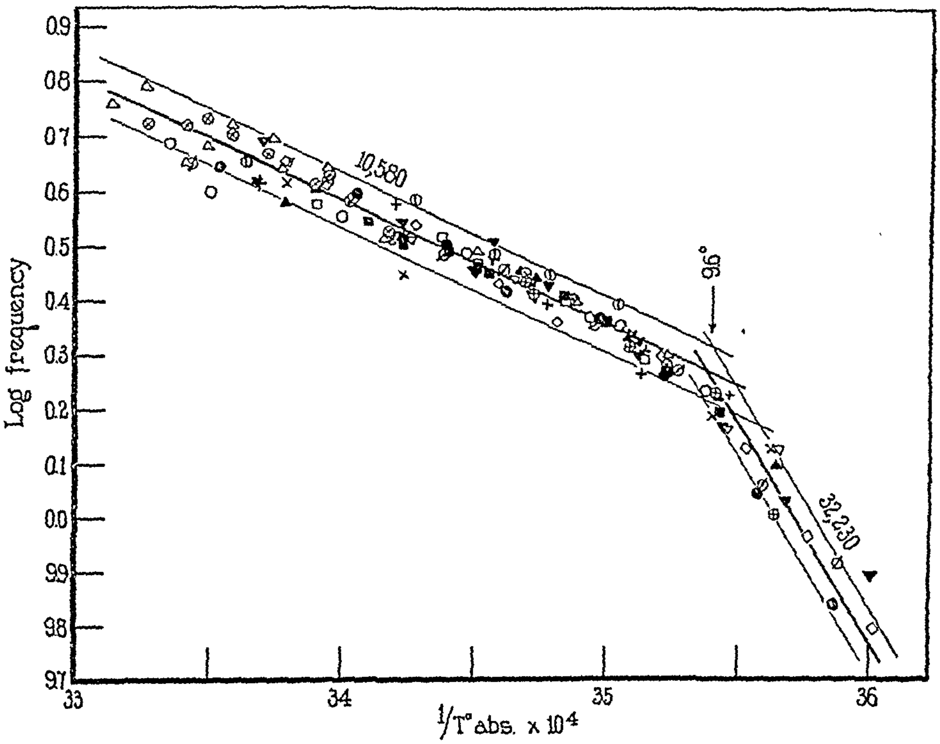

FIG. 1. Observations on the frequency of the contraction of the umbrella of *Gonionemus*. Data from 18 normal animals, at temperatures between 4° and 28°C., for which μ = 10,580 between 9.6° and 28°C. and μ = 32,230 between 4° and 9.6°C.

III.

The 29 "normal" individuals used for the measurement of the frequency of the contraction must be subdivided into three groups, according to the μ values and the critical temperatures which they show. Most of the animals give μ = 10,580 calories between 29° and 10°C.; below 10°C. μ = 32,230. The break is rather sharp at a temperature of 9.6°C. These data are collected in Fig. 1.

A second group of normal animals gives μ = 8,160 above 14°C. and

22,510 below 14°. Finally, 3 animals were found which could not be brought into either of the preceeding two groups. The value of μ for these, taken together, was found to be 11,420 above 12.3°C. and 22,800 below about 12°C.

Figs. 1, 2 and 3 give the plots of the results obtained in these experiments. On comparing the μ values with those already known as typical it is seen that they are similar to values found in a great many other cases and that the critical temperatures happen to be located at places where they are typically found (*cf.* Crozier, 1925–26, *a*, *b*).

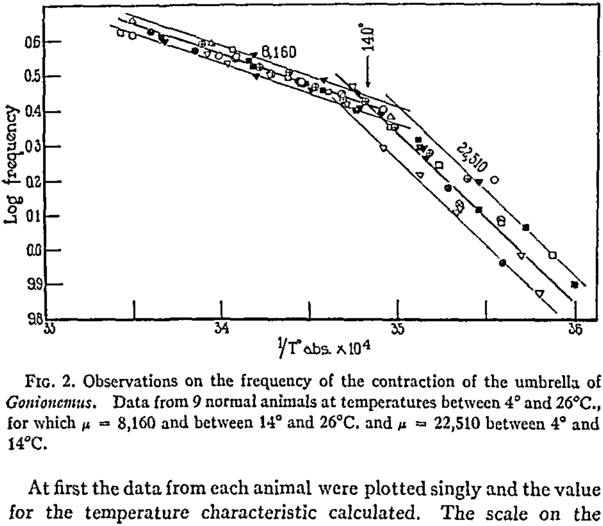

FIG. 2. Observations on the frequency of the contraction of the umbrella of *Gonionemus*. Data from 9 normal animals at temperatures between 4° and 26°C., for which μ = 8,160 and between 14° and 26°C. and μ = 22,510 between 4° and 14°C.

At first the data from each animal were plotted singly and the value for the temperature characteristic calculated. The scale on the ordinate was chosen very large, therefore even the largest scatter of points represents not more than 1 second difference in the pulsation frequency. According to the values found, and to the location of the "breaks," the animals were brought into the different groups into which they clearly fell. The rate of pulsation in the different individuals is not exactly the same, so that in bringing the groups together the frequency has to be multiplied by a certain small factor in each

case, which changes the relative frequency but not the slope of the line and hence not the μ values. After having brought the different groups together in this way the location of a break is easier to determine precisely (because of the larger number of observations) and the mean value for the temperature characteristics is easily found. Special attention has to be paid to these regions of the plots located around a "break," where one finds a "rounding off" in such a way

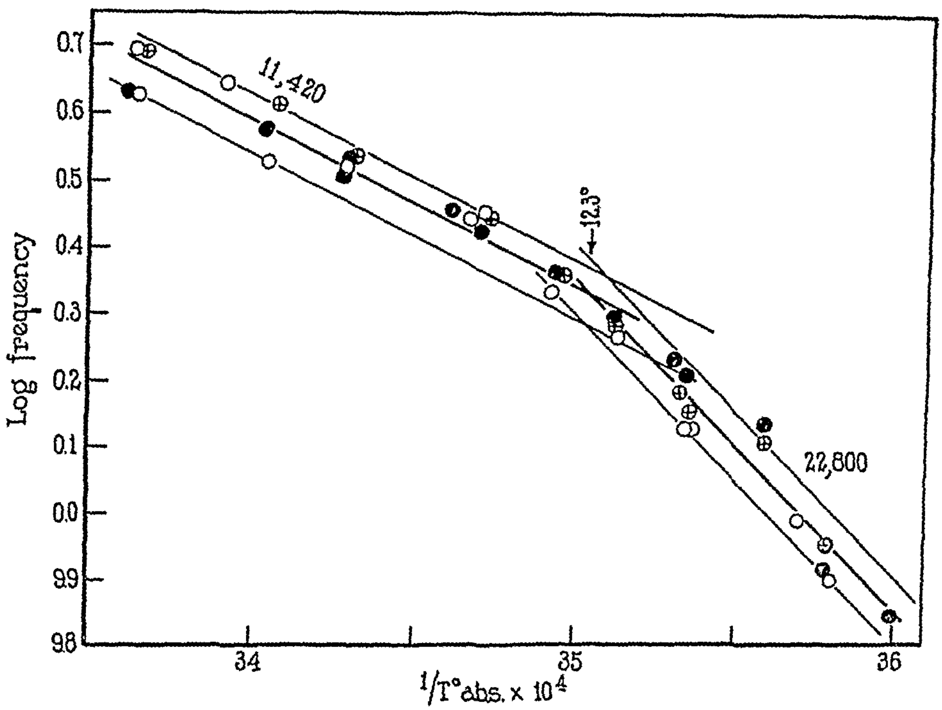

FIG. 3. Observations on the frequency of the contraction of the umbrella of *Gonionemus*. Data of 3 normal animals at temperatures between 4° and 24°C., for which μ = 11,420 between 12.3° and 24°C. and 22,800 between 4° and 12.3°C.

that most of the points fall nearer to the lower margins of the best fitting ribbons (Figs. 1 and 2). This shows that in going up or down the temperature scale the single readings in the neighborhood of the break may belong sometimes to the higher, sometimes to the lower μ value. Other reasons for this "rounding off" have been mentioned in earlier papers (Crozier and Stier, 1926–27, *a*, *b*, *c*). Furthermore, it is to be noticed that in all cases when animals were found constituting a separate group the number of individuals was always greater than

one, and that they recurred at different times during the course of the summer.

IV.

In the second class there are placed the results obtained from 17 animals where the nerve ring was cut in two places, on opposite sides of the margin of the umbrella. This operation has in most of the cases no effect on the rate of pulsation at room temperature. In several cases, however, the beat becomes irregular and retarded for the 1st hour after the operation, which is indicated in the plot (Fig. 7) by a number of points which fall off the normal band at the beginning of the several "runs." Later the pulsation becomes as regular as in normal animals, so that the points fall near together and adhere well to the line describing the course of all. The animals used can be placed together in one group. The μ value for temperatures between 29° and 13.4°C. = 10,510 calories, and below 13.4° = 21,380. The value for the higher temperature range is the same as for the first group of the normal animals. For lower temperature range we notice a difference, since the animals with two marginal cuts give μ = 21,380 calories, but the normals 32,000. Furthermore, attention has to be paid to the fact that in this case the "break" occurs at 13.4°C., in the case of the "normals" at 9.6°C.

In the second group of the "normals", however, the "break" occurred at 14°C., which corresponds sufficiently well to the result for the animals with two cuts.

In the same way, animals with only one cut and others with four cuts were used. The few experiments with animals having one marginal cut were incomplete, but the μ value is approximately the same as in the experiments with the animals with two cuts.

Only 2 animals with four cuts were used. In the activity there is no difference, in general, when compared with the animals used before. The μ value calculated for a high temperature range = 10,660 and for lower temperatures = 21,000, the break occurring at about 13.1°C. These values correspond well with the values for the individuals with two marginal cuts.

Looking over the results of these experiments, it can be said that we get for all the animals, for the temperature range above 15°C., only

two different values for the temperature characteristics, *i.e.*, μ = 11,000 $\pm$ and μ = 8,000 $\pm$; for lower temperatures we get μ = 21,000 $\pm$ and in one group μ = 32,000. These values are closely comparable with values well established in earlier experiments. The critical temperatures, also, are located at places where they have been found before.

The operation of cutting through the nerve ring might help to show whether the contraction of the umbrella is controlled by only one pace maker or by more. On the supposition that we have to deal with more than one pace maker, it is to be expected that we should get different μ values for animals treated in the same way; or better, we should expect the points as plotted to be largely "sprayed" in the form of a fan, which would indicate that sometimes the contraction is under the control of one pace maker and then under the control of another one for which supposedly different μ values might be obtained. This expectation was not realized. The μ values are rather constant for animals after the different operations.

Finally, there is to be added to the foregoing groups one more, where the medusæ were cut in half. Only four such preparations were used. The contraction of the umbrella becomes rather irregular after the operation but later becomes sufficiently uniform. The beat is occasionally irregular; and the animals may keep quiet for a long time, in which cases they were touched with a glass rod to activate them. The value for μ found for a temperature range between 19° and 4°C. is 16,900, without evidence of any break. In view of the fact that only four half animals were used and not enough points were gotten for the plots, no particular weight can be put on this result, which nevertheless is of interest for the subsequent discussion.

V.

For the remaining experiments the nerve ring was entirely cut off, in 22 animals. After the operation the medusæ stop beating for a long interval, beating recovers but there is a difference between the beating and that in the groups already considered. The contraction of the umbrella is more quick and violent, and the time between two beats accordingly longer. Furthermore, ten beats, one after another, occur only seldom; in many cases only five, six or eight beats can be

counted in a continuous group. The animals are not particularly active, so they had to be touched often with the glass rod; after stimulating in this way the first two or three beats are irregular. Measurements were taken as soon as the beats became regular again. The results of the observations are given in the plot of Figs. 4 and 5. The animals can obviously be arranged in two groups. The first one (8 cases) gives a μ value of 16,000 for the whole temperature range, without break. The second group (10 cases) shows μ = 8,100 above 15°C.,

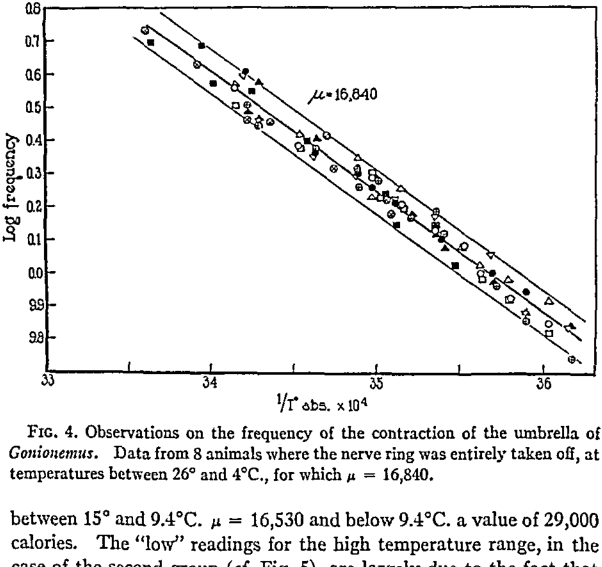

FIG. 4. Observations on the frequency of the contraction of the umbrella of *Gonionemus*. Data from 8 animals where the nerve ring was entirely taken off, at temperatures between 26° and 4°C., for which μ = 16,840.

between 15° and 9.4°C. μ = 16,530 and below 9.4°C. a value of 29,000 calories. The "low" readings for the high temperature range, in the case of the second group (*cf.* Fig. 5), are largely due to the fact that apparently the readings were taken too soon after the operation, when the animals were not yet sufficiently recovered. The points are rather irregularly spread over a large range. The value for the middle part corresponds to that for the first group but for the lower range we get a different value from that for the first group. The break is located on

the same place as with the "normal" animals and with the animals having two cuts. Looking over the first group of those without nerve ring we find, however, that in some animals perhaps a slight indication of a break, or of irregularity, can be found at 10°C., but it is not so certain that the animals can be taken out of the μ = 16,000 group.

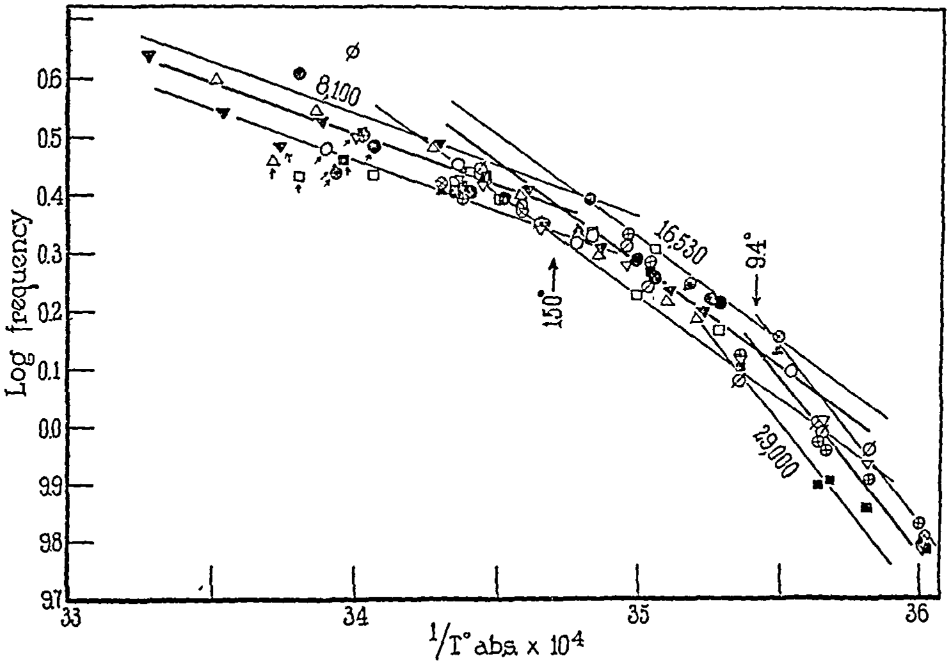

FIG. 5. Observations of the frequency of the contraction of the umbrella of *Gonionemus*. Data from 10 animals in which the nerve ring was taken off. The μ value for a temperature range between 15° and 26°C. = 8,100; for temperatures between 15° and 9.4°C. = 16,530; and μ = 29,000 for temperatures between 9.4° and 4°C.

VI.

The fact that not all the normal animals show the same increments nor the same critical temperatures is parallel to earlier findings in other cases (Crozier and Stier, 1924–25, *c*; 1926–27, *a,c*). On the other hand it does seem true that where different μ's appear for the same activity in the same kind of organism, either without deliberate experimental interference (and hence "normally," as it would usually be said), or as result of direct operation or treatment, the increments found appear to belong to a consistent system (Crozier and Stier, 1924–25, *a*).

The respiratory phenomena of insects and other animals provide μ = 8,000±; 11,000; 16,000±; 32,000. So also in the present instance, these increments are found, combined in various ways, and with the addition of 22,000± for certain individuals at lower temperatures. The interrelations between these values, as in *Notonecta* (Crozier and Stier, 1926–27, *a*), do not encourage the notion that the 2:1 ratio sometimes suggested by the μ magnitudes above and below an intermediate critical temperature is in itself of any significance.

The different classes of "normal" medusæ are not obviously distinguishable on the basis of known accidents of weather, time, size or relative frequency of pulsation. They correspond to those internally existing metabolic differences which have been appealed to in the case of the hearts of embryonic *Limulus* (Crozier and Stier, 1926–27, *c*). It is noteworthy that in no single case is there evidence of the fan-wise distribution of the logarithmic pulsation frequencies, as was conceived possible before the experiments began. The definiteness of the μ classes should make it profitable to investigate in detail the means whereby individuals in one class may be made to exhibit other increments.

Removal of the marginal ring effects profound changes in the thermal relations of pulsation frequency. Two classes of individuals appear. In one, comprising 11 cases, μ = 16,500 in the mid-region, 29,000± at low temperatures, 8,100± (but irregular) at high, breaks coming at 15° and at 9.4°. In the other, with 8 individuals, μ = 16,800 without break. The absolute rates of pulsation are different before and after the operation, being slower when the marginal ring has been removed. The logarithmic latitude of variation, it is to be noted, is sensibly identical (*cf.* Figs. 1, 4 and 5); this is of interest in relation to the question of a possible interconnection between critical increment and variability of rate. The differences between medusæ with and without marginal ring are so unmistakable that it is difficult or indeed impossible to view the determination of the critical increments in the "normal" individuals as controlled by processes pertaining to the musculature or to the conducting nerve net of the bell. They are obviously to be attributed to the marginal sensoria. It should also be observed that if muscular processes (*i.e.* relaxation viscosity), independently affected by temperature, were superimposed on

the effect of the sense organs we might look for a curvilinear connection between log F and $1/T$, as found for the speed of progression of tent caterpillars (Crozier and Stier, 1925–26, *a*) and seen also in data of analogous sort from other sources.

The difference between the results of Figs. 4 and 5 are not to be attributed to differences in time elapsing after the operation. Some medusæ in each group were cut 2 hours, or as much as 24 hours, before the experiments began, and the readings were checked by return observations. Therefore the difference must be a real one, although the scatter of the points in Fig. 5, above 15°, suggests the "fan" effect sought when these experiments began; in fact, however, the divergent points (indicated by arrows) were obtained at the beginning of "runs," and are taken to be due to faulty thermal adaptation, which disappears by the time the experiment has gotten well under way. Although the technical procedure with these individuals did not differ from that followed with those in Fig. 4, the presence of the break at 9.4° independently marks these individuals as distinct, and as requiring for best results a more careful handling (although this could not be foreseen while the tests were being made). In a number of instances an individual of each group (Figs. 4 and 5) was under observation at the same time in the same thermostat, so gross technical differences are excluded. The line for μ = 8,100 in Fig. 5 is drawn with the initial observations omitted; its significance is slight, except as fixing the critical temperature at 15° in agreement with inspection. The position at which critical temperatures occur are, for the normal medusæ, 9.6° (12.3°), 14° for those deprived of sensoria, 9.4°, 15° (Fig. 5), or not at all (Fig. 4). This agreement is striking, and may be held to signify that the critical temperatures are not determined by properties of the sensoria, but belong to the rest of the pulsation complex, even in normal medusæ.

The effect of removal of the sensoria of *Gonionemus* is curiously parallel to that obtained in connection with the pulsatile cloaca of holothurians (Crozier, 1916; Crozier and Stier, unpublished experiments) in which the cloacal apparatus gives in the intact animal μ = 12,200 for frequency of contraction, but after amputation μ = 20,000 (*cf.* Crozier, 1916). One difference between these cases is the persistence of members of the "series" of increments 8,000; 11,000±;

16,000; 30,000. These might be compared with known increments for nerve net processes (*cf.* Crozier and Pilz, 1923–24; Crozier, 1924–

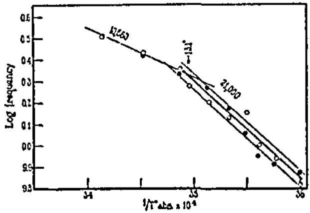

FIG. 6. Observations of the frequency of the contraction of the umbrella of *Gonionemus*. Data from 2 animals in which the nerve ring was cut in four places. For temperatures above 13.1°C. μ = 10,660; and below 13.1°C. μ = 21,000.

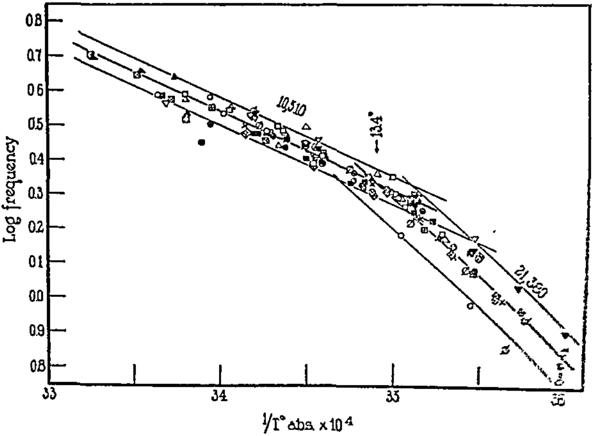

FIG. 7. Observations on the frequency of the contraction of the umbrella of *Gonionemus*. Data from 17 animals in which the nerve ring was cut in two opposite sides of the margin of the bell. For temperatures above 13.4°C. μ = 10,510 and below 13.4°C. μ = 21,380.

25, *b*; and other measurements since available), and without serious disagreement; but with echinoderms the data on the holothurian cloaca show that no significance can be given to the values of μ as diagnostic for types of tissues (Crozier and Stier, 1924–25, *c;* 1925–26, *b;* 1926–27, *c;* 1927–28).

If this conception be correct, of different mechanisms for control of the frequency of initiation of pulsation as determinable by marginal sense organs and by the rest of the pulsating complex, it ought to be possible to produce a variety of centers of origin for contractions by making cuts in the marginal ring. This experiment is a necessary preliminary to tests in which all but 1 or 2 sensoria are to be removed. It was

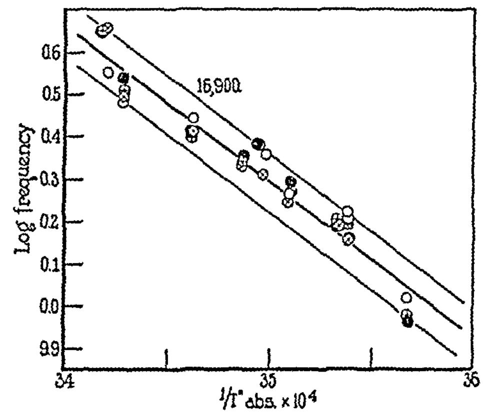

FIG. 8. Observations on the frequency of the contraction of the umbrella of *Gonionemus*. Data from 4 animals which were cut in half. The μ value for the whole temperature range = 16,900 (see text).

carried out in two groups of medusæ, in one of which (2 animals) four equally spaced radial incisions were made, so far as possible through the ring merely (not damaging the velum); in the other group (17 animals) two diametrically opposite cuts were made. All the animals so treated agree in showing μ = 10,500± above 13°–15°, μ = 21,000± below (Figs. 6 and 7). One pretty definitely indicated feature of these measurements is the increased, but constant, relative latitude of variation below 15°. This type of change of logarithmic latitude associated with a change of μ has interesting possibilities for the general theory of these studies, as has been previously indicated (*cf.* Crozier and Stier, 1926–27, *b*).

It is a curious fact that among the normal animals (Figs. 1 and 2) the values $\mu = 10,580$ and $\mu = 32,230$ are found associated, with the critical temperature 9.6°, and in the other group $\mu = 8,160$ and $\mu = 22,500$, with critical temperature 14°; in the present cases $\mu = 10,500$ is associated with 21,000±, the "break" being at 14°−, and the increased relative latitude of variation is also unquestionably connected with $\mu = 22,000$. The experiments plotted in Figs. 6 and 7 were made at different times during the summer, and the differences from the results with normal medusæ cannot be attributed to seasonal changes, nor to any obvious differences such as might be associated with size or time of confinement in aquaria. The values of μ are sufficiently definite to show that the operation of merely dividing the nerve ring does not bring about the state of affairs obtained when the whole nerve ring is removed (Figs. 4 and 5). Hence a final attempt to produce animals in which a conflicting mixture of critical increments might be expected to appear was made by cutting medusæ in half. In this way we might look for an intermixing of pulsations originating both from (1) marginal sense organs and from (2) nerve net along the zone of cutting. The visible contractions begin on the bell margin. The data obtained (Fig. 8) show that although the variability is increased above that in uncut animals, the increment is pretty definitely 16,000±, without breaks, as in the majority of cases without nerve ring (Fig. 4).

SUMMARY.

The frequency of contraction of the bell of *Gonionemus* was studied in relation to temperature, with intact animals and also where different operations were made on the nervous system. A number of values of μ are found for intact animals namely 8,100±, 10,500±, 32,000± and 22,500±, with critical temperatures at 9.6°, 12.3°, and 14.0°. Four different classes of operations were used: (1) Animals where the nerve ring was cut on two opposite sides of the bell; the μ values found are 10,500± and 21,300±, with a critical temperature at 13.4°. (2) Animals with four cuts through the nerve ring gave $\mu = 10,600$ ± and $\mu = 21,000$, with a critical temperature at 13.1°. (3) In animals where the bell was cut in half the temperature characteristic was found to be 16,900. And finally (4) in the animals where the nerve ring was

totally removed μ values of 8,100, 16,000±, and 29,000 were found, with critical temperatures at 15.0° and 9.4°.

These results are discussed from the standpoint of the theory which supposes that definite "temperature characteristics" may be associated with the functional activity of particular elements in a complex functional unit, and that these elements may be separately studied and identified by suitable experimental procedures involving the magnitudes of the respective temperature characteristics and the locations of associated critical temperatures. The swimming bell of medusæ with its marginal sense organs permits a fairly direct approach to such questions. It is found that even slight injuries to the marginal nerve ring, for example, produce specific modifications in the temperature relations which are different from those appearing when the organism is cut in half.

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PHOTIC ORIENTATION BY TWO POINT-SOURCES OF LIGHT.

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I.

It has been shown (Crozier, 1925–28) that negatively phototropic organisms are oriented by beams of parallel light rays opposed at 180° according to the equation

$$\tan \theta = \cot \frac{H}{2} \cdot \frac{(I_1 - I_2)}{(I_1 + I_2)},$$

where I_1 and I_2 are intensities (luminous flux per unit area normal to the rays), H the head angle, and θ the angle between the normal to the light rays and the position of orientation. It was also shown that a positively or negatively phototropic organism is oriented by beams of parallel light rays at an angle of 90° according to the equation

$$\tan \theta = \frac{I_2 \tan H/2 - I_1}{I_1 \tan H/2 - I_2},$$

where θ is the angle between one of the beams (I_1 for photonegative, I_2 for photopositive forms) and the position of orientation. It was pointed out (Crozier, 1925–28) that these equations may be derived from a more general expression. It is desirable to examine the general case with some care in order to provide a basis for subsequent experiments.

These formulæ, as well as those employed by various other investigators (*cf.* the previous paper), may be shown to be special cases of a general equation for orientation with two point-sources of light at a finite distance apart. The general equation may take either of two forms, depending on the type of organism and the method of experimentation. The simpler case is that in which the organism does not

change its position with relation to the sources of light by any forward locomotion, but merely assumes a position of orientation by turning about an axis perpendicular to the plane on which it rests. This will be termed the problem of orientation *in situ*. The orientations of sessile forms or of motile forms which are attached to the substratum in some manner fall into this group of phenomena; of this type are the experiments of Northrop and Loeb (1922–23) with *Limulus* attached by the tail so that forward progression did not occur.

The more complicated case is that in which the animal moves continuously along a curved path, being influenced at every point by the two light sources. This will be termed the problem of orientation *in transitu*. The case of curvilinear orientation by a single source of light presents additional features (Crozier and Stier, 1927–28, *a*) which may require recognition when orientation *in transitu* is controlled by two sources.

The problem of orientation *in situ* will be considered first.

II.

The generalized animal with which the equations propose to deal is considered as consisting of a body with two photoreceptive surfaces at the anterior end, equally inclined to the axis of the body and of equal area (Fig. 1). The angle h between a photoreceptive surface

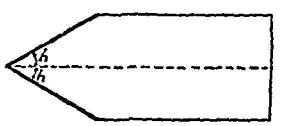

FIG. 1. The generalized phototropic animal considered in the derivation of equations for orientation; h is the half head angle.

and the body axis will be termed the *half head angle*. The head angle H of Crozier's paper (1925–28) is equal to $2h$. In animals such as the larva of the blow-fly (*Calliphora erythrocephala*) h is apparently constant (Crozier, 1925–28; Patten, 1914). In young rats before the eyes are open (Crozier and Pincus, 1926–27), and in other forms, the head angle varies as a function of the light intensities used. The possibility of variation in the head angle shows that this angle is not entirely a structural matter, but is in some cases at least partially

dynamic, depending on the movement of the head from side to side. Similar cases arise in geotropism (Crozier and Stier, 1927–28, *b*).

The field in which the animal is placed is considered to be a horizontal surface with the two point-sources of light practically in the plane of the surface. A system of Cartesian coordinates bears the following relation to the lights: The X axis passes through the lights; the Y axis bisects the distance between the lights. The coordinates of the lights (L' and L'') are $(-a, 0)$ and $(a, 0)$ respectively. The animal at any moment has its anterior end at the point A (x, y). The angle of orientation, θ, is the positive angle between the X axis and the axis of the animal. The distances from the animal to the lights (AL' and AL'') are m and n respectively, and are given in terms of x, y, and a by the ordinary distance formula, as

$$m^2 = (a + x)^2 + y^2,$$

$$n^2 = (a - x)^2 + y^2.$$

The dimensions of the photoreceptive surfaces are considered very small in relation to m and n, and the variations in the values of m and n and the angles which the light rays make with the photoreceptive surfaces at different parts of the surfaces are negligible.

The lights L' and L'' have *luminous intensities* of P' and P'' *candles*, respectively.

The photochemical effect on the photoreceptive surface may be considered as proportional either to the total luminous flux falling on it, or to the logarithm of this amount. For the case of the animal oriented *in situ* (or, with parallel rays, *in transitu*) it makes no difference which condition is assumed (*cf.* Crozier, 1925–28), as will be seen later.

III.

We will first consider the problem of the negatively phototropic animal with constant head angle oriented *in situ*. The animal will always turn so that its anterior end is away from the X axis. Orientation is possible only in the limited field enclosed in a circle passing through the lights L' and L'', in which the minor arc $L'L''$ is measured by a central angle equal to $4h$ (Fig. 2). In this circle the lines joining

any point on the major arc $L'L''$ (such as A) with L' and L'' form an angle at A equal to $2h$, and the planes of the photoreceptive surfaces

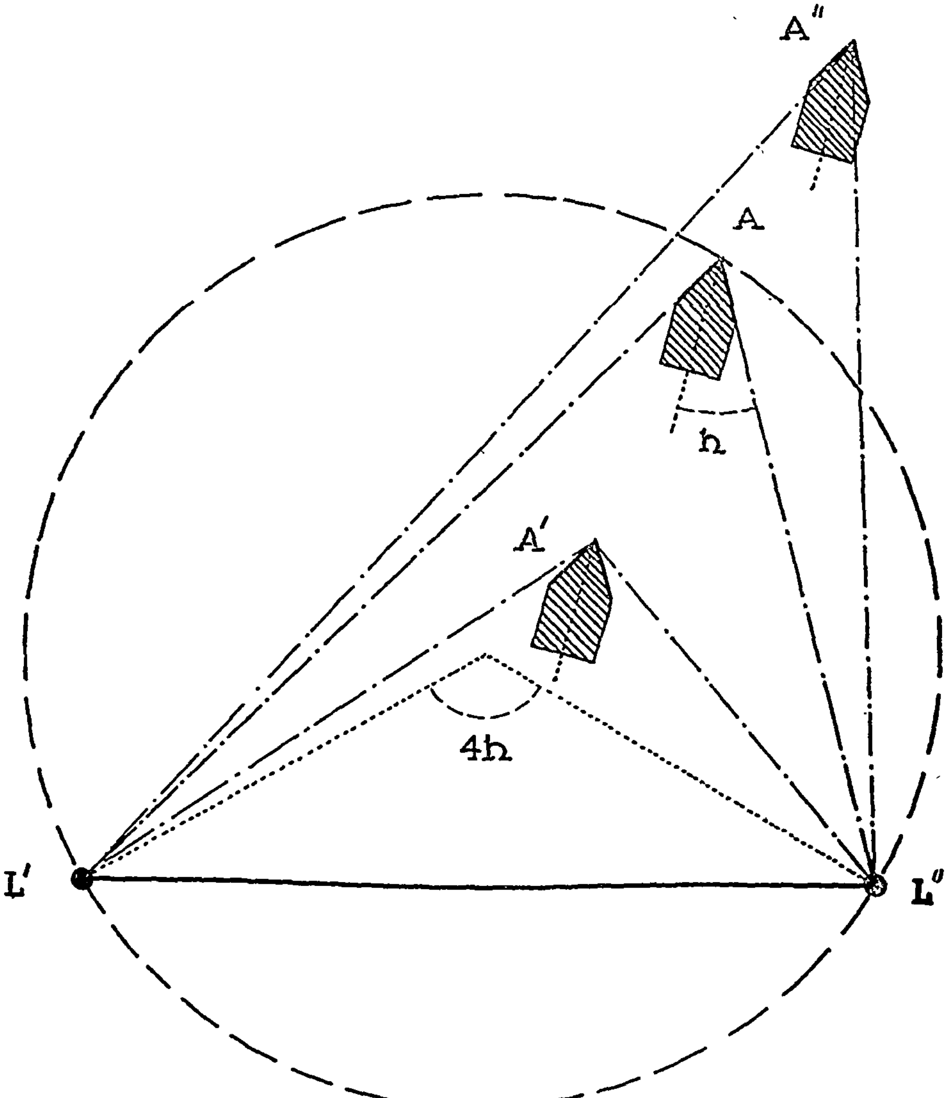

FIG. 2. The negatively phototropic organism can be oriented by two point-sources, within a circle passing through the lights L' and L'' with the minor arc $L'L''$ intercepting a central angle equal to $4h$.

therefore pass through the lights. At any point inside the circle (A') the photoreceptive surfaces may both be affected by the lights. At

any point outside the circle (A'') not more than one photoreceptive surface may be affected at a time.

The problem, then, is to obtain the angle θ in terms of x, y, a, h, P', and P''. We will define the angle α as the angle at which rays from L' strike the left photoreceptive surface, and the angle β as that at which the rays from L'' strike the right photoreceptive surface (Fig. 3).

Orientation will be accomplished and maintained when the animal is in such a position that the photochemical effects on the two photoreceptive surfaces are equal (Northrop and Loeb, 1922–23). The

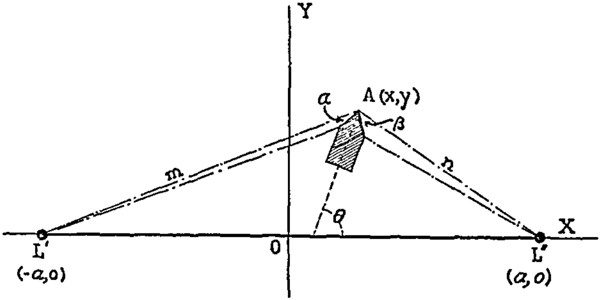

FIG. 3. The photonegative organism at A (x, y) oriented by point-sources of light L' ($-a$, o) and L'' (a, o).

effect, as before stated, may be assumed proportional either to the incident luminous flux ($E = kF$) or to its logarithm ($E = k \log F$).

At the position of orientation E' equals E'', and consequently F' equals F'', whether the direct or the logarithmic relation be assumed. If B is the area of each photoreceptive surface,

$$F' = \frac{P' B \sin \alpha}{m^2}$$

and

$$F'' = \frac{P'' B \sin \beta}{n^2};$$

whence, at a position of complete orientation,

$$\frac{P' n^2}{P'' m^2} = \frac{\sin \beta}{\sin \alpha}. \tag{1}$$

$$\theta = \alpha + h + \widehat{\sin}\, \frac{y}{m},$$

$$\alpha = \theta - h - \widehat{\sin}\, \frac{y}{m},$$

$$\sin \alpha = \sin (\theta - h) \frac{(\alpha + x)}{m} - \cos (\theta - h) \frac{y}{m}$$

$$= \frac{1}{m} [(a + x) (\sin \theta \cos h - \cos \theta \sin h) - y (\cos \theta \cos h + \sin \theta \sin h)].$$

$$\pi = \beta + h + \theta + \widehat{\sin}\, \frac{y}{n},$$

$$\beta = \pi - \theta - h - \widehat{\sin}\, \frac{y}{n},$$

$$\sin \beta = \sin (\pi - \theta - h) \left(\frac{a - x}{n}\right) - \cos (\pi - \theta - h) \frac{y}{n}$$

$$= \frac{1}{n} [(a - x) (\sin \theta \cos h + \cos \theta \sin h) - y (- \cos \theta \cos h + \sin \theta \sin h)].$$

Substituting in (1) and dividing numerator and denominator by *cos θ cos h*, we have

$$\frac{P'n^2}{P''m^2} = \frac{\frac{1}{n} [(a - x) (\tan \theta + \tan h) + y (1 - \tan \theta \tan h)]}{\frac{1}{m} [(a + x) (\tan \theta - \tan h) - y (1 + \tan \theta \tan h)]}.$$

Clearing fractions and writing $t = \tan h$,

$$P' n^3 [(a + x) (\tan \theta - t) - y (1 + t \tan \theta)]$$

$$= P'' m^3 [(a - x) (\tan \theta + t) + y (1 - t \tan \theta)],$$

or

$$\tan \theta = \frac{P' n^3 [t (a + x) + y] + P'' m^3 [t (a - x) + y]}{P' n^3 [a + x - ty] - P'' m^3 [a - x - ty]}. \tag{2}$$

This is the required expression, since m and n are given as functions of x, y, and a.

IV.

The problem of the positively phototropic animal oriented *in situ* presents slightly different conditions. The animal is now oriented with its anterior end *toward* the X axis. There are two cases, which will be considered separately.

Case I.—The animal is in a position in which light from one of the sources can strike only one of the photoreceptive surfaces.

Case II.—The animal is in a position in which light from both sources strikes each photoreceptive surface.

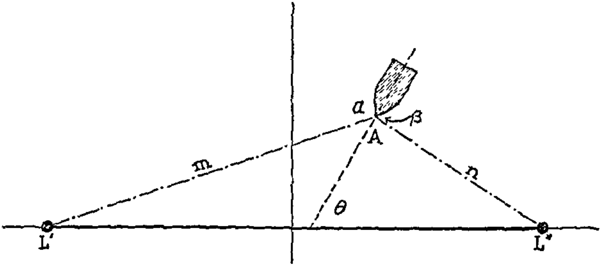

FIG. 4. The photopositive organism at A oriented by point-sources of light L' and L''. Case I: Light from each source strikes only one photoreceptive surface.

V.

Case I. (Fig. 4).

The quantities m and n are defined as before. Since the animal is now in the reverse position to that in Fig. 3 the angles α and β now refer to the opposite sides of the animal although to the same lights as before.

At the position of orientation, equation (1) must again hold.

$$\alpha = \pi - \theta - h + \widehat{\sin}\,\frac{y}{m},$$

$$\sin \alpha = \sin (\pi - \theta - h) \frac{(a + x)}{m} + \cos (\pi - \theta - h) \frac{y}{m}$$

$$= \frac{1}{m} [(a + x) (\sin \theta \cos h + \cos \theta \sin h) - y (\cos \theta \cos h - \sin \theta \sin h)].$$

$$\beta = \theta - h + \widehat{\sin} \frac{y}{n},$$

$$\sin \beta = \frac{1}{n} [(a - x) (\sin \theta \cos h - \cos \theta \sin h) + y (\cos \theta \cos h + \sin \theta \sin h)].$$

Substituting in (1) and again dividing by *cos θ cos h*, and writing $t = \tan h$, we have

$$\frac{P'n^3}{P''m^3} = \frac{(a - x) (\tan \theta - t) + y (1 + t \tan \theta)}{(a + x) (\tan \theta + t) - y (1 - t \tan \theta)},$$

from which

$$\tan \theta = \frac{P'n^3 [t (a + x) - y] + P''m^3 [t (a - x) - y]}{- P'n^3 [a + x + ty] + P''m^3 [a - x + ty]}. \tag{3}$$

VI.

Case II. (Fig. 5).

In this case the animal is at such a distance from the lights that both sides of the head are affected by both lights. In addition to α and β, which are defined as before, we have an angle γ at which the rays from L' strike the left photoreceptive surface and an angle δ at which the rays from L'' strike the right photoreceptive surface.

The luminous flux on the right photoreceptive surface is now

$$F' = \frac{P' B \sin \alpha}{m^2} + \frac{P'' B \sin \gamma}{n^2}$$

and that on the left is

$$F'' = \frac{P'' B \sin \beta}{n^2} + \frac{P' B \sin \delta}{m^2};$$

whence, at the position of orientation,

$$\frac{P'n^2}{P''m^2} = \frac{\sin\beta - \sin\gamma}{\sin\alpha - \sin\delta}. \tag{4}$$

$$\alpha = \pi = \theta - h + \widehat{\sin}\,\frac{y}{m},$$

$$\beta = \vartheta - h + \widehat{\sin}\,\frac{y}{n},$$

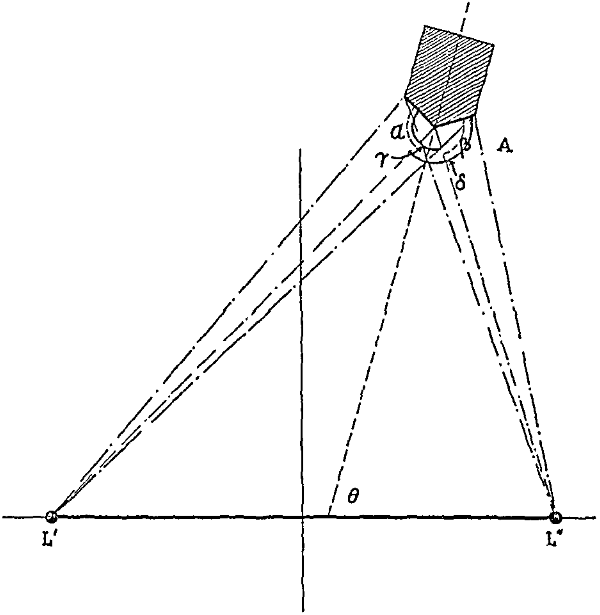

FIG. 5. The photopositive organism at A oriented by point-sources of light L' and L''. Case II: Light from each source strikes both photoreceptive surfaces.

$$\gamma - \alpha = \delta - \beta = \pi - \widehat{\sin}\,\frac{y}{m} - \widehat{\sin}\,\frac{y}{n},$$

$$\gamma = 2\,\pi - \theta - h - \widehat{\sin}\,\frac{y}{n} = -\left(\theta + h + \widehat{\sin}\,\frac{y}{n}\right),$$

$$\delta = \pi + \theta - h - \widehat{\sin}\,\frac{y}{m};$$

$$\sin\alpha = \frac{1}{m}\left[(a+x)\,(\sin\theta\cos h + \cos\theta\sin h) - y\,(\cos\theta\cos h - \sin\theta\sin h)\right],$$

$$\sin\beta = \frac{1}{n}\left[(a-x)\,(\sin\theta\cos h - \cos\theta\sin h) + y\,(\cos\theta\cos h + \sin\theta\sin h)\right],$$

$$\sin\gamma = -\left[\sin\,(\theta+h)\,\frac{(a-x)}{n} + \cos\,(\theta+h)\,\frac{y}{n}\right]$$

$$= -\frac{1}{n}\left[(a-x)\,(\sin\theta\cos h + \cos\theta\sin h)\right.$$

$$\left. + \, y\,(\cos\theta\cos h - \sin\theta\sin h)\right],$$

$$\sin\delta = \sin\,(\pi+\theta-h)\,\frac{(a+x)}{m} - \cos\,(\pi+\theta-h)\,\frac{y}{m}$$

$$= \frac{1}{m}\left[(a+x)\,(-\sin\theta\cos h + \cos\theta\sin h)\right.$$

$$\left. + \, y\,(\cos\theta\cos h + \sin\theta\sin h)\right].$$

Substituting in (4), dividing by $\cos\theta\cos h$, and writing $t = \tan h$,

$$\frac{P'n^3}{P''m^3} = \frac{[(a-x)\,(\tan\theta - t) + y\,(1 + t\tan\theta) + (a-x)\,(\tan\theta + t) + y\,(1 - t\tan\theta)]}{[(a+x)\,(\tan\theta + t) - y\,(1 - t\tan\theta) - (a+x)\,(-\tan\theta + t) - y\,(1 + t\tan\theta)]}$$

$$= \frac{2\,(a-x)\tan\theta + 2\,y}{2\,(a+x)\tan\theta - 2\,y}.$$

Clearing fractions,

$$\tan\theta\,P'n^3\,(a+x) - P'n^3\,y = \tan\theta\,P''m^3\,(a-x) + P''m^3\,y,$$

whence

$$\tan\theta = \frac{y\,(P'n^3 + P''m^3)}{P'n^3\,(a+x) - P''m^3\,(a-x)}. \tag{5}$$

It is here seen that when conditions are such that Case II applies, the position of orientation is independent of h.

VII.

Parallel rays of light act as if coming from a point infinitely distant. To apply any of these equations to orientation by beams of parallel rays we set $m = n$ and $x = 0$; y and a are infinite, but the ratio y/a is finite and may be expressed as a function of some angle. Under these conditions equation (2) becomes

$$\tan\theta = \frac{(P' + P'')\,(at + y)}{(P' - P'')\,(a - ty)}$$

$$= \frac{(P' + P'')\,(t\tan\psi/2 + 1)}{(P' - P'')\,(\tan\psi/2 - t)},$$

where ψ is the angle $L'AL''$. Equation (3) becomes under the same conditions

$$\tan\theta = \frac{(P'' + P')\,(t\tan\psi/2 - 1)}{(P'' - P')\,(\tan\psi/2 + t)},$$

and equation (5)

$$\tan\theta = \frac{P' + P''}{P' - P''}\cot\psi/2.$$

When parallel light rays are used the organism orients independent of its position in the field. Orientation *in transitu* with parallel rays therefore leads to motion along a straight line, there being no further turning once the position of orientation is reached. The equations for orientation *in situ* will thus apply equally well to the case of orientation *in transitu* when beams of parallel rays are used.

VIII.

The special case of (negatively phototropic) animals oriented by beams of parallel light rays opposed at 180° is described by setting $x = 0$, $y = 0$, and $m = n$ in equation (2):

$$\tan\theta = \frac{P'at + P''at}{P'a - P''a} = t\,\frac{P' + P''}{P' - P''}.$$

Since with parallel rays the intensity at all points is constant, $I = k P$. θ as we here define it is $90° - \theta$ as Crozier (1925–28) used it, and calling his angle θ' we get by substitution

$$\tan \theta' = \cot \theta = \frac{1}{t} \frac{P' - P''}{P' + P''}, \text{ or}$$

$$\tan \theta' = \cot h \frac{I' - I''}{I' + I''}. \tag{6}$$

IX.

The special case of light beams of parallel rays at right angles and a positively phototropic organism is described by substituting $x = 0$, $y = a$, $m = n$ in equation (3).

$$\tan \theta = \frac{P' (at - a) + P'' (at - a)}{- P' (a + at) + P'' (a + at)}$$

$$= \frac{(P' + P'') (1 - t)}{(P' - P'') (1 + t)}.$$

Our angle θ here equals $45° + \theta$ as used by Crozier (1925–28), which we will term θ'';

$$\theta'' = \theta - \frac{\pi}{4}$$

$$\tan \theta'' = \frac{\tan \theta - 1}{\tan \theta + 1} = \frac{(P' + P'') (1 - t) - (P' - P'') (1 + t)}{(P' + P'') (1 - t) + (P' - P'') (1 + t)}$$

$$= \frac{P'' - P' t}{P' - P'' t},$$

and since our L' corresponds to I_2 in Crozier's figure and our L'' to his I_1, then

$$\tan \theta'' = \frac{I_2 \tan h - I_1}{I_1 \tan h - I_2}$$

as previously found.

If h is equal to or greater than 45° it can easily be seen that equation (5) must hold, and we then have

$$\tan \theta = \frac{P' + P''}{P' - P''},$$

or substituting θ'', I_1, and I_2

$$\tan \theta'' = \frac{\tan \theta - 1}{\tan \theta + 1} = \frac{P''}{P'} = \frac{I_1}{I_2}$$

(*cf.* Section VI; and Crozier, 1925–28).

X.

For the case of negatively phototropic animals oriented by beams of light at right angles (as in the experiments of Loeb and Northrop (1917) with *Balanus* larvæ), substituting $x = 0$, $y = a$, $m = n$ in equation (2) we find

$$\tan \theta = \frac{P'(at + a) + P''(at + a)}{P'(a - at) - P''(a - at)}$$

$$= \frac{(P' + P'')(1 + t)}{(P' - P'')(1 - t)};$$

or, writing $\theta'' = \theta - 45°$,

$$\tan \theta'' = \frac{\tan \theta - 1}{\tan \theta + 1} = \frac{(P' + P'')(1 + t) - (P' - P'')(1 - t)}{(P' + P'')(1 + t) + (P' - P'')(1 - t)}$$

$$= \frac{P'' + P' t}{P' + P'' t},$$

or in terms of I_1 and I_2—

$$\tan \theta'' = \frac{I_2 \tan h + I_1}{I_1 \tan h + I_2}.$$

XI.

We have considered h as constant throughout these equations. It will be practically constant under such experimental conditions that the total intensity of the lights is maintained constant. Its value for different intensity ranges may be determined by equation (6).

When the photoreceptive surfaces are parallel, h is of course equal to zero. Equation (2) becomes in this case

$$\tan \theta = \frac{(P'n^2 + P''m^2)\, y}{P'n^2 (a + x) - P''m^2 (a - x)},$$

and equation (3)

$$\tan \theta = \frac{(P'n^3 + P''m^3)\, y}{P'n^3\,(a + x) - P''m^3\,(a - x)}.$$

XII.

When L' and L'' have equal luminous intensities P' and P'' drop out of the equations, equation (2) becoming

$$\tan \theta = \frac{n^3\,[t\,(a + x) + y] + m^3\,[t\,(a - x) + y]}{n^3\,[a + x - ty] - m^3\,[a - x - ty]},$$

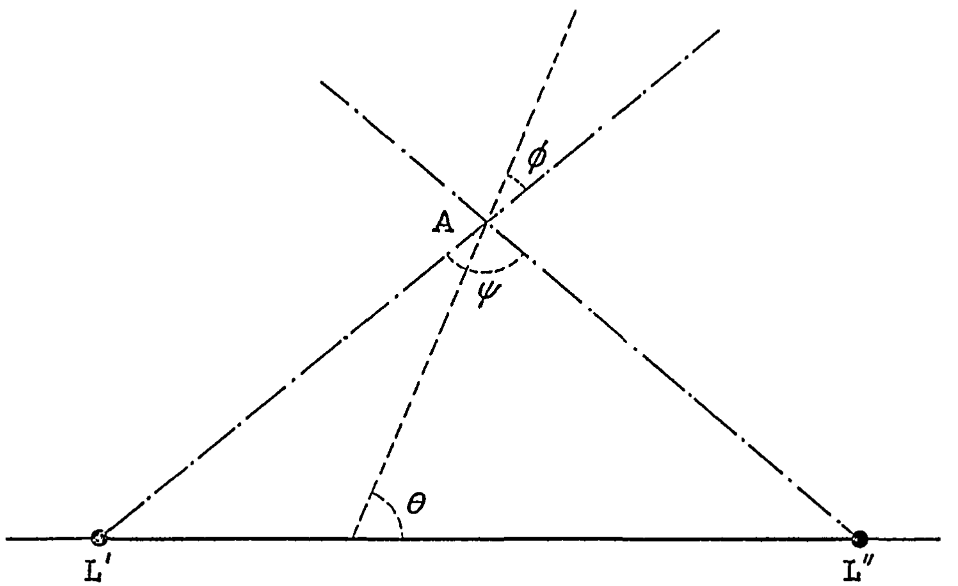

FIG. 6. Relation between θ, ψ, and Buder's angle ϕ.

equation (3) becoming

$$\tan \theta = \frac{n^3\,[t\,(a + x) - y] + m^3\,[t\,(a - x) - y]}{-\,n^3\,[a + x + ty] + m^3\,[a - x + ty]},$$

and equation (5) becoming

$$\tan \theta = \frac{y\,(m^3 + n^3)}{n^3\,(a + x) - m^3\,(a - x)}.$$

XIII.

The experiments of Buder (1917–19) with negatively phototropic *Chlamydomonas* and *Carteria* illustrate orientation with beams of

parallel rays at an oblique angle. The head angle in this case may be considered equal to zero (*cf.* Crozier, 1925–28).

Setting $t = 0$ in the equation for beams of parallel rays (Section VII) we have

$$\tan \theta = \frac{P' + P''}{P' - P''} \cot \frac{\psi}{2} = \frac{R + 1}{R - 1} \cot \frac{\psi}{2},$$

where R is the ratio P'/P''. Buder's angle ϕ is equal to $\theta - (\pi/2 - \psi/2)$ (see Fig. 6).

$$\tan \phi = \tan (\theta - \pi/2 + \psi/2) = - \frac{1}{\tan (\theta + \psi/2)}$$

$$= \frac{\tan \theta \tan \psi/2 - 1}{\tan \theta + \tan \psi/2} = \frac{\frac{R+1}{R-1} - 1}{\frac{R+1}{R-1} \cot \psi/2 + \tan \psi/2}$$

$$= \frac{2}{R\left(\cot \frac{\psi}{2} + \tan \frac{\psi}{2}\right) + \left(\cot \frac{\psi}{2} - \tan \frac{\psi}{2}\right)} = \frac{2}{R \frac{2}{\sin \psi} + \frac{2 \cos \psi}{\sin \psi}}$$

$$= \frac{\sin \psi}{R + \cos \psi}.$$

Computing the expected values of ϕ by this equation we check the experimental results very closely, and somewhat better than by the method of vectors which Buder employed. The algebraic sum of the "errors" was -7.0° with Buder's computed values, but only -1.27° with ours.

XIV.

The problem of an animal orienting *in transitu* presents a different situation. The path of such an animal from a point then depends on the direction in which it is moving when at that point. Fig. 7 shows the paths of a blow-fly larva away from a fixed point in the plane. The straight dotted lines represent the animal's paths before the lights were turned on. When the animal was at A the lights were turned on and its paths thereafter are indicated by the solid lines. The numerals refer to order in which the trails were made. The

lights were small "Spotlight" bulbs about 1 cm. above a ground glass plate which served as the creeping plane. It is seen that the paths pursued by the larva are quite different in the different trails, although all starting at the same point.

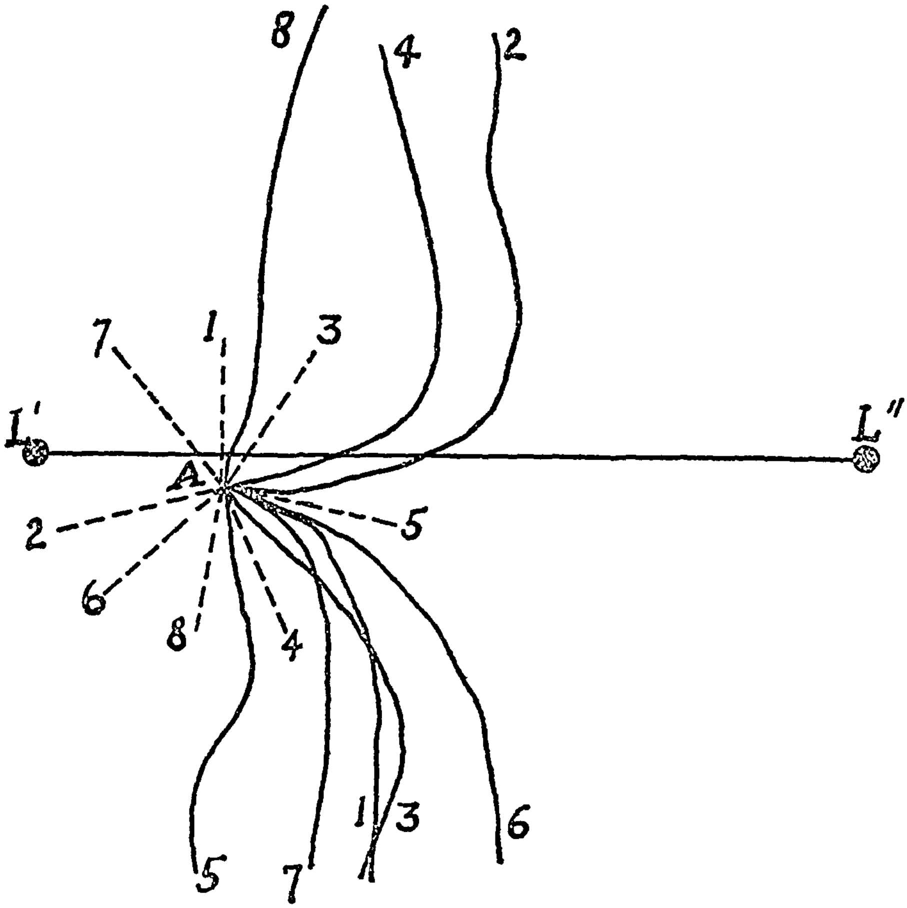

FIG. 7. Trails of a blow-fly larva oriented *in transitu* by point-sources of light L' and L''; L' L'' equals 20 cm. The animals crept toward point A $(-5.5, -0.5)$ under the influence of a weak directing light bulb (dotted lines). When at A the directing light was turned off and L' and L'' turned on. The animal then moved along a curved path (solid lines) until outside the field of influence (*cf.* Section III, Fig. 2) of the light. Numerals (1–1, 2–2, etc.) indicate successive trails.

The photochemical processes in the photoreceptive surfaces affect the rate at which the slope of the path varies with time (or with respect to distance traversed, since the velocity of creeping may be assumed constant).

The rate of change of slope may be written $\frac{d\theta}{dt}$, or $\frac{d\theta}{ds} \cdot \frac{ds}{dt}$, in which the second factor is the constant velocity. θ is the angle the tangent to the path at any point makes with the X axis (Fig. 8).

It has not as yet been determined whether for such instances the rate of turning should be considered proportional to the difference between the effects on the two sides of the animal or to their ratio, but it is probable that either one or the other of these conditions holds.

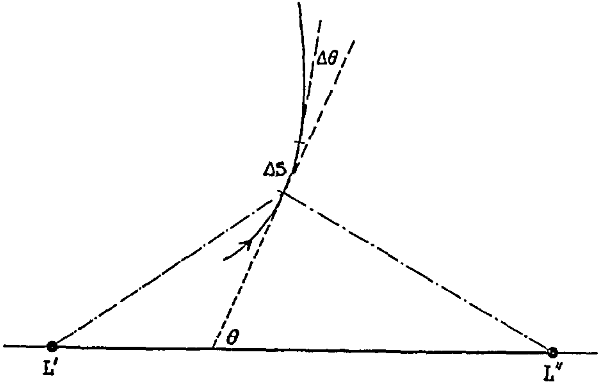

FIG. 8. Path of a photonegative animal oriented *in transitu;* Δs is an element of path, $\Delta\theta$ an element of angle.

That is, either $\frac{d\theta}{dt} = K \frac{E'}{E''}$ or $\frac{d\theta}{dt} = K\ (E' - E'')$. Since at the same time we do not know whether E is effectively proportional to F or to *log* F, there are four possible statements of the problem, which are

$$\left.\begin{array}{ll} \text{(i)}\ \frac{d\theta}{dt} = K_1 \frac{F'}{F''}; & \text{(ii)}\ \frac{d\theta}{dt} = K_2 \frac{\log F'}{\log F''}; \\ \text{(iii)}\ \frac{d\theta}{dt} = K_3\,(F' - F''); & \text{(iv)}\ \frac{d\theta}{dt} = K_4 \log\,(F'/F''). \end{array}\right\} \quad (7)$$

Writing $Q_1 = \dfrac{d\theta}{dt} \cdot \dfrac{1}{K_1}$, etc.,

$$Q_1 = F'/F''$$

$$Q_2 = \frac{\log F'}{\log F''}$$

$$Q_3 = F' - F''$$

$$Q_4 = \log \left(\frac{F'}{F''}\right).$$

If we confine our attention to the case of negatively phototropic animals (Fig. 2) equation (7, i) may be developed thus:

$$Q_1 = \frac{P'n^2 \sin \alpha}{P''m^2 \sin \beta}$$

$$= \frac{P'n^3 \left[(a + x)(\tan \theta - t) - y(1 + t \tan \theta)\right]}{P''m^3 \left[(a - x)(\tan \theta + t) + y(1 - t \tan \theta)\right]}.$$

Equation (7,ii) gives

$$Q_2 = \frac{\log P' - 2 \log m + \log \sin \alpha}{\log P'' - 2 \log n + \log \sin \beta},$$

of which further development leads into a rather bad mathematical morass. Equation (7, iii) gives

$$Q_3 = \frac{P' \sin \alpha}{m^2} - \frac{P'' \sin \beta}{n^2}$$

$$= \frac{P'}{m^3}\left[(a + x)(\tan \theta - t) - y(1 + t \tan \theta)\right] - \frac{P''}{n^3}\left[(a - x)(\tan \theta + t) + y(1 - t \tan \theta)\right]$$

$$= \tan \theta \left[\frac{P'}{m^3}(a + x - ty) - \frac{P''}{n^3}(a - x - ty)\right] - \left\{\frac{P'}{m^3}\left[t(a + x) + y\right] + \frac{P''}{n^3}\left[t(a - x) + y\right]\right\};$$

and (7, iv)

$$Q_4 = \log \frac{P'n^3\,[(a+x)\,(\tan\theta - t) - y\,(1 + t\tan\theta)]}{P''m^3\,[(a-x)\,(\tan\theta + t) + y\,(1 - t\tan\theta)]}.$$

All these equations give $\dfrac{d\theta}{dt}$ in terms of the coordinates of the animal and of the direction in which it is moving. Experiments

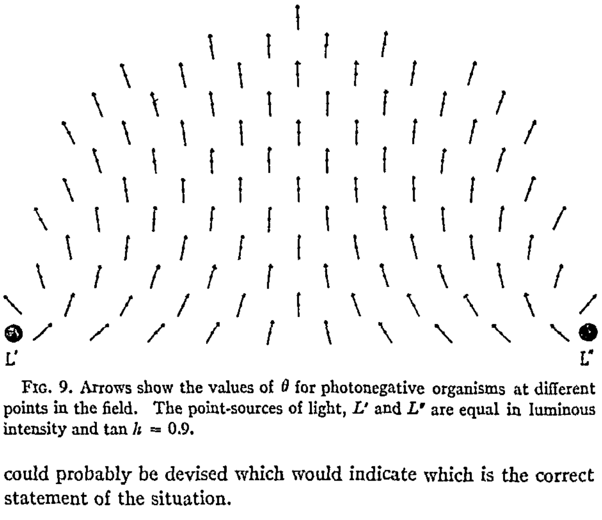

FIG. 9. Arrows show the values of θ for photonegative organisms at different points in the field. The point-sources of light, L' and L'' are equal in luminous intensity and $\tan h = 0.9$.

could probably be devised which would indicate which is the correct statement of the situation.

XV.

When the velocity $\dfrac{ds}{dt}$ is small, orientation is very nearly complete at every point on the path. When it is zero the animal is motionless as far as progression is concerned, and we have the problem of orientation *in situ*.

If we may regard $\dfrac{ds}{dt}$ as so small that the animal is practically

oriented at every position, equation (2) gives the equation of the path of the animal, by writing

$$\frac{dy}{dx} = \frac{P'n^3\,[(a + x)\,t + y] + P''m^3\,[(a - x)\,t + y]}{P'n^3\,[a + x - ty] - P''m^3\,[a - x - ty]}$$

and eliminating the derivative. Since m and n are both complicated functions of x, y, and a, the expression is probably unsolvable as a differential equation. The integration may be done graphically, however, and the animal's path plotted; or the field may be mapped out as a field of force with arrows showing the values of θ at various points. Such a field is plotted in Fig. 9, P' and P'' being equal and t being given the value 0.9, which is its approximate value for blow-fly larvæ.

XVI.

It has been shown (*cf.* Yagi, 1927–28) that illumination of part of the compound eye of certain arthropods (*e.g.*, *Dixippus*) may produce effects that vary for the different parts. The results of experiments with point-sources of light would be invalidated by this condition since a point-source would affect very few of the ommatidia and the effect would be different for different parts of the eye, depending on the angle which the axis of the animal made with the light rays.

It is quite impossible to have a light of sufficient brilliance that will be an actual point, and the nearer a light is to the animal the greater the angle it will subtend and the more ommatidia it will affect. The number of ommatidia affected will then also be a determining factor in the direction of orientation. Parker (1903) found that positively phototropic butterflies (*Vanessa*) in a room would fly to a comparatively large window admitting a diffuse light rather than to a much more brilliant artificial light within the room.

The difficulty presented by these differences in effect in different parts of the eye may be circumvented in such animals as *Limulus* by providing each of the compound eyes with a rigid hood having a window covered by a diffusing screen. The window acts as a source of light of constant area and fixed position with respect to the eye. Then the intensity alone varies as the animal moves in relation to the light sources. It might also be pointed out that the value of the head

angle can be artificially fixed by this apparatus, since the angle is now that between the planes of the two windows.

XVII.

Regarded simply as the orientation of a machine with bilaterally disposed photoreceptors, the description of phototropic behavior which may aim at some degree of quantitative comprehensiveness is seen to present a very fair degree of complexity. This at once becomes apparent if we seek to predict the character and extent of orientation when more than one source of light is effective, on the basis of what is known regarding phototropism in the simplest kinds of situations. It should be noted, therefore, that inability to deal quantitatively with the measurable aspects of the orientation of organisms in compound fields of illumination is not to be taken gratuitously as evidence of supramechanical influence at work within the organism. In an elementary way the kinds of complication revealed by the present analysis serve to demonstrate concretely the *justification for quantitative investigation under the most rigidly* simplified conditions attainable.

XVIII.

SUMMARY.

General formulæ are derived for the orientation of phototropic organisms in a field illuminated by two point-sources of light in the same horizontal plane.

It is shown that formulæ previously found may all be derived as special cases of these general equations.

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INTRACELLULAR OXIDATION-REDUCTION STUDIES.

I. Reduction Potentials of Amœba dubia by Micro Injection of Indicators.*

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INTRODUCTION.

The reductive power of living cells, tissues and bacterial cultures, as evidenced by their action on colored indicators, has been the subject of much study since the observation by von Helmholtz in 1843. With the passage of years there has accumulated a large amount of evidence, more or less contradictory in some respects but quite unanimous as to the cardinal fact that the living cell exerts a truly remarkable reducing power. In general, the earlier observers, and a number of recent ones, made no sharp distinction between the intensity of reduction, the quantity and *time*, which are the dimensions of reducing *power*.

It was not until 1920 that Gillespie presented data suggesting that the reducing intensity of bacterial cultures might be measurable in terms of electrode potential. Shortly thereafter Clark (1920), by measuring the equilibrium potentials between methylene blue and methylene white and between indigo blue and indigo white, gave quantitative values to the different reduction intensities indicated by these systems; and at about the same time (1920) he also presented a comprehensive basis for interpreting, in terms of electrode potential, the results given by the biological reduction of reversible oxidation-reduction systems. This pioneer work opened the way to the study and elaboration at the Hygienic Laboratory of a number of reversible oxidation-reduction indicators. The assortment of com-

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pounds now available is, as we shall see, by no means exhaustive nor entirely satisfactory; but it appears to be adequate for a preliminary survey of certain limited aspects of the biological problem.

Following the hint given by Gillespie, a considerable amount of work has been done by Clark and Cohen (1922, 1925, 1928) in an effort to correlate the reduction potentials attained in bacterial cultures and cell suspensions with certain phases of metabolism and life history. Some of the results have been summarized in the paper by Cannan, Cohen and Clark (1926). In this contribution a general correlation was shown between the reduction potential of a cell suspension, the cellular reduction of a particular dye and the reduction potential of the same dye as determined in pure solution. It was also found that cell suspensions are poorly poised with respect to the electromotively active material present at any moment; but that active material is slowly mobilized by cell activity from some large reserve, and the electromotive activity is paralleled by a reductive activity toward appropriate indicators.

A more direct attack upon the problem of cellular reduction is recorded in the admirable studies made by Needham and Needham (1925, 1926). They were the first to inject reversible oxidation-reduction indicators into living cells and to observe microscopically the color changes undergone by these compounds. In observations on *Amœba proteus* they report that this cell is capable not only of reducing the oxidized forms of indicators of more positive potential but also of oxidizing the leuco form of indicators of oxidation-reduction potential lower than its own. This led to the conclusion that this organism is able to maintain a fairly constant reduction potential at a zone lying somewhere between rH 17 and 19.[1] Needham and Needham claimed that when the ameba was studied in atmospheres of nitrogen, hydrogen or oxygen, they could discover no essential change in the picture, and they therefore concluded that the rH of the ameba is probably widely independent of the concentration of oxygen in the external atmosphere.

Other organisms and cells were studied by these authors and by Rapkine and Wurmser (1926, 1927), and additional support was adduced for the thesis that each type of cell maintains a more or less characteristic and independent internal reduction potential.

This announcement of the existence of a fairly well poised oxidation-reduction system within the cell stands in contrast to the conditions found by Cannan, Cohen and Clark in bacterial cultures which showed an increasing reduction intensity as the neutralizing effect of oxygen was eliminated. A further study of the problem therefore seemed appropriate.

In the experiments to be described we sought to learn how far the

[1] The reader is referred to the papers by Clark and Cohen for discussion of the elementary aspects of reversible oxidation-reduction equilibria.

microinjection technic in its present stage of development could be applied to the problem, and to what degree we would repeat and possibly extend the observations of the Needhams upon the ameba. To this end each of a series of indicators, in its oxidized or reduced state, was injected under controlled conditions of oxygen access. It was also of importance to determine the relative toxicity of the indicators, to provide a guide for purification of the compounds and for the synthesis of new ones.

EXPERIMENTAL.

Amebæ.—The experiments reported in this paper were upon *Amœba dubia* principally. In a subsequent communication experiments will be reported on certain marine ova, fertilized and unfertilized. A number of observations were made upon *A. proteus* and the results were always in agreement with those obtained with the other species. We confined our attention largely to *A. dubia* because its interior is less granular and its pellicle is more easily pierced by the micro pipet.

The amebæ were supplied by Dr. J. A. Dawson of Harvard University; as soon as received, the original culture was diluted with glass-distilled water to which were added 2 or 3 boiled wheat grains. The cultures were stored in the ice box and were thus maintained in excellent condition.

The cells were washed once with distilled water and usually placed in groups of about 4 to 8 in hanging drops. We found it convenient to mark off with paraffin wax two rows of contiguous circles on the cover-slip and thus to keep separate different lots of cells as well as drops of reagents and wash water.

The internal pH of both *A. dubia* and *A. proteus* has been found to be 6.9 ± 0.1 by Chambers, Pollack and Hiller (1927), who state that these cells maintain this internal pH as long as they are in normal condition, and that they possess an appreciable buffering capacity. Significant change in internal pH is associated with death of the cell. Thus the natural buffering ability of the protoplasm made it unnecessary to employ buffered dye solutions for injection. Some injections were tried with indicator dissolved in buffer, but disturbing salt effects were encountered. We shall see that even in aqueous solution, certain compounds produced salt effects due to inorganic ions occasionally present as impurities.

Injection Apparatus.—The injections were made with the improved double micromanipulator apparatus (Chambers, 1928). One manipulator carried a micro needle to move the ameba into proper position and to hold it in place and the other carried a micro pipet for injecting the cell.

The apparatus with a straight pipet, described by Chambers (1922–23), was employed for injections made in the presence of air.

For injections under strict anaerobiosis, a moist chamber was used which was provided with a mercury seal; in this case the shank of the pipet was given a

U-shape (see Fig. 1) to dive under the mercury seal in the manner described by Barber (1914) and by Needham and Needham (1926).

The whole system, from the syringe to the shank of the micro pipet, was filled with deaerated water; only a small air space was left in the pipet proper. The oxygen contamination from this source was eliminated later when necessary in the sealed chamber by replacing the air with the gas of the chamber and washing the pipet several times with the injection fluid.

The hermetic moist chamber for injections under anaerobiosis was made of glass, the joints being cemented together with balsam (see Fig. 1). It consisted of two troughs, one to hold mercury for sealing the chamber and the other to serve as the moist chamber proper. The latter contained a gas inlet and an outlet. Through the axis of the inlet there passed a capillary tube used for delivering the reduced dye into the moist chamber under complete exclusion of oxygen. The chamber was 30 × 40 mm., and 15 mm. high; and the total volume, including the gas space over the mercury seal, was about 25 cc. Purified nitrogen was passed through at a rate varying from 50 to 250 cc. per minute. The chamber was roofed over by a cover-slip (40 × 60 mm.) and sealed by strips of mica, all heavily coated with vaseline and pressed firmly on the broad contact surfaces. The seal was tested for tightness and found to withstand a pressure of at least 2 to 3 cm. of water. The diffusion of oxygen through the vaselined seals was of a negligible order, or else it was effectually eliminated by the constant stream of purified nitrogen passing through the chamber, for drops of reduced indicator often remained uncolored on the cover-glass for several hours during an experiment.

Purification of Nitrogen.—An essential part of the experimental technic is the particular care to eliminate leakage of atmospheric oxygen into the anaerobic system. Unprotected rubber connections are dangerous because oxygen diffuses through the rubber in quantities sufficient to upset conditions and lead to erroneous conclusions. This point needs emphasis.

Commercial Linde process nitrogen containing about 0.5 per cent oxygen was used. The oxygen was removed by passing the gas through an electrically heated (between 650° and 750°C.) Pyrex glass tube, filled with copper gauze and wire which had been previously reduced with hydrogen. The issuing stream of purified nitrogen was moistened by passage through a bubbler of distilled water. The gas was conveyed through narrow copper tubing to the moist chamber. All joints in the gas line were of metal-to-metal or glass-to-metal, the latter being sealed with deKhotinsky cement. Some difficulty occurred in sealing the joint at the moist chamber for it was necessary to maintain a flexible and sliding tight fit for the few moments when a drop of reduced dye was delivered to the cover-glass. This was finally solved by cracking the seal for a moment (with nitrogen flushing the system), depositing the drop of dye on the slide and quickly resealing the joint. By providing a long, well greased sliding fit at this joint, the latter may be safely left without further sealing.

Indicators and Reagents.—The amebæ were injected with the compounds listed

in Table I. For convenience in designating and referring to the indicator compounds in our list, we have assigned to each a letter as shown in the table. Opposite each compound is given its characteristic E_0' value (the potential on the hydrogen scale) at pH 7.0, that is, the electrode potential difference between the normal hydrogen electrode and an equimolecular mixture of oxidant and reductant at pH 7.0. These values may be converted into rH values which are independent of pH. The compounds are thus placed on a graduated scale of reducing (or oxidizing) intensity irrespective of their other chemical characters. The potentials of the hydrogen and theoretical oxygen and air electrodes are included as orienting reference points. Compounds on the positive end of the scale are "more oxidizing" than those on the negative end; and conversely, those on the negative end are "more reducing."

In addition to the indicators, there is included in this scale the ferricyanide system, because we have depended in critical experiments upon injections of potassium ferricyanide to restore the color of intracellularly reduced dyes and thus to make sure that the compound under investigation was still present in the cell and potentially available. We have found that small and moderate[2] injections of aqueous 1 per cent potassium ferricyanide are tolerated by the normal ameba. Large injections cause the cell to round up and take on a yellow color; the granules sink in the cytoplasm and the ameba becomes motionless and dies. At no time have we found the freshly made ferricyanide solution to produce a blue coloration within an ameba which had not been treated with a blue dye. This point is emphasized for we are aware of the possibility of the formation of Prussian blue and similarly colored iron compounds. The results were frequently checked by noting the effect of air or of potassium chromate on the reduced dye within the cell. Instances of apparent disagreement proved to be due to a time factor involved; because the dye was possibly undergoing decomposition and one of the tests was performed soon enough to give a positive result while the next test was done too late. In other words, injection of fresh ferricyanide promptly after decoloration of the reversible indicators always restored the color of the dye or intensified it; if the oxidizing agent was injected long after the dye had been reduced, the color might or might not be restored depending upon secondary effects.

The oxidation-reduction indicators used were 25 in number, all but one of which were synthesized at the Hygienic Laboratory. Phenosafranin was a fairly pure commercial product (Safranin B extra, Badische). Three sulfonated indophenols (compounds *A1*, *A2* and *B* in the tables) were specially synthesized[3] to test the validity of Dixon's (1926) suggestion that the toxicity of the sulfonic acid might yield a false index of the reducing ability of the cell. There is also included in this group compound *M*, a disulfonated indophenol. The list contains two indicators, phenol blue (dimethylaniline indophenol) and *m*-toluylene diamine

[2] See p. 593.

[3] We wish to acknowledge our indebtedness to Mr. W. L. Hall of the Hygienic Laboratory who made these compounds for us.

indophenol, amphoteric compounds which have been studied by Cohen and Phillips but which have not yet been reported. Finally there is the group comprising the last three compounds in the tables, namely, neutral red, dimethylaminomethylphenazine and phenosafranin. These are rather unsatisfactory for pur-

TABLE I.

List of Compounds Injected.

	Name of oxidant	E_0' at pH 7.0 (volts)	rH
	(Oxygen electrode)	+0.81	41.0
	(Oxygen in air)	+0.80	40.7
	Potassium ferricyanide	+0.43	28.4
	Potassium chromate	?	?
A1	Phenol *m*-sulfonate indo-2,6-dibromophenol	0.273	23.1
B	Phenol *m*-sulfonate indophenol ... approx.	0.25	22.4
C	*m*-Bromophenol indophenol	0.248	22.3
A2	Phenol *o*-sulfonate indo-2,6-dibromophenol	0.235	21.9
D	*o*-Chlorophenol indophenol	0.233	21.8
E	*o*-Bromophenol indophenol	0.231	21.7
F	Phenol blue chloride	0.227	21.6
G	Bindschedler's green zinc chloride	0.224	21.5
H	Phenol indo-2, 6-dichlorophenol	0.217	21.3
I	" " " dibromophenol	0.217	21.3
J	*m*-Cresol indophenol	0.210	21.0
K	*o*-Cresol "	0.195	20.5
L	*o*-Cresol indo-2, 6-dichlorophenol	0.181	20.1
M	*1*-Naphthol-2-sulfonate indophenol *m*-sulfonic acid	0.135	18.5
N	*m*-Toluylene diamine indophenol chloride	0.127	18.3
O	*1*-Naphthol-2-sulfonate indophenol	0.123	18.1
P	*1*-Naphthol-2-sulfonate indo-2,6-dichlorophenol	0.119	18.0
Q	Toluylene blue chloride	0.115	17.9
R	Methylene blue chloride	+0.011	14.4
S	K_4 indigo tetrasulfonate	−0.046	12.5
T	K_3 " trisulfonate	−0.081	11.3
U	K_2 " disulfonate (also Na_2)	−0.125	9.9
V	Neutral red iodide ... approx.	−0.30	4.0
W	Dimethylaminomethylphenazine chloride	?	?
	(Hydrogen electrode)	−0.421	0.0
X	Phenosafranin ... approx.	−0.525	−3.5

The potential and rH values listed are those found at 30°C. Our experiments were performed at room temperatures ranging from about 20° to 25°; the small corrections applicable have been ignored for present purposes.

poses of studying the cell potential because they do not seem to possess, in the physiological range of pH, the labile reversibility demanded by our experiments. They were included, however, for such incidental information as they might yield.

Preparation of Reduced Dye Solutions.—The apparatus for reducing the indicator solutions and storing them is shown in Fig. 1. The dye was dissolved, filtered if necessary, and then placed in the reducing flask *F* together with washed, platin-

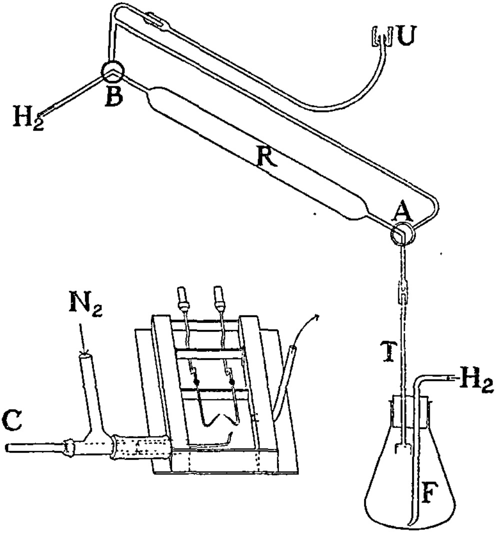

Fig. 1.

ized asbestos. Moist hydrogen was then bubbled through the suspension until the dye was decolorized. Meanwhile the escaping hydrogen passed through the filter tube *T* and the reservoir *R* and washed out the oxygen contained in them. The filter tube *T* was provided at its lower end with a perforated platinum disk upon which was laid a thin mat of washed asbestos. Its upper end was connected with the inlet to the reservoir. When the dye was reduced, the flask *F* was raised, the filter tube *T* sliding down through the rubber stopper until its lower end reached the flask bottom. The pressure of hydrogen then forced the reduced in-

dicator solution through the filter and up into the reservoir. When filtration was completed, the cocks *A* and *B* were turned so as to isolate the reservoir from the exterior, and *T* was detached from the reservoir.

To remove residual hydrogen purified nitrogen was bubbled through the solution in the reservoir. In the case of photosensitive compounds like leucomethylene blue, the reservoir was covered with black paper.

If furnished with properly ground and well greased cocks, the reservoir will preserve the solutions indefinitely. There is still on hand, at this writing, one containing reduced naphthol sulfonate indophenol which was prepared in July, 1927, and which shows not the slightest visible trace of change.

To deliver the reduced dye to the moist chamber, the procedure is as follows: The moist chamber is thoroughly deaerated with purified nitrogen as indicated by the arrows. Meanwhile the reservoir is fixed (*A* upward and *B* down) in close proximity to the moist chamber and is joined to the nitrogen supply by a small gas-tight, bronze union *U*. Nitrogen is now allowed to flush through cocks *A* and *B* alternately until the system, excepting *R*, is deaerated. The lower outlet (from cock *B*) is then joined by a short rubber connection to the outer end (*C*) of the capillary delivery pipet leading into the moist chamber, and the delivery pipet is deaerated.

This rubber connection is only a temporary one and is eliminated as a source of oxygen contamination by our subsequent procedure. After blowing nitrogen through the capillary for 15 to 30 minutes for thorough deaeration, cock *A* is turned to open the reservoir to the nitrogen supply, and cock *B* is carefully turned to let reduced dye solution run slowly through the capillary delivery tube. The solution washes forward any residual oxygen contamination from the short rubber connection, and the first few drops of solution are wasted on absorbent paper placed for this purpose along the back and bottom of the moist chamber.

The wasted dye solution serves still another purpose, for it acts as an oxygen absorbent in the chamber. We may state that with ordinary careful technic even the first drop of the entering solution of reductants, such as leucomethylene blue and leuco-indigo carmine, was entirely devoid of tell tale signs of reoxidation.

The tip of the capillary delivery tube is then raised and a drop of clear reductant deposited on the under side of the cover-glass of the moist chamber. During all this manipulation, a steady stream of nitrogen is running through the chamber. As soon as the drop of reductant is delivered to the slide, the wax seal (cracked while depositing the reductant) is restored and cock *B* is shut off. Since the capillary delivery tube remains filled with reductant, any subsequent infiltration of oxygen through the rubber connection is effectually neutralized. In fact, the rubber connection may be detached and the end of the delivery tube *C* safely left exposed to the air, for the rate of oxygen diffusion in the capillary is exceedingly low. Leaks in the chamber are quickly betrayed by the coloring of the indicator drop; we have frequently kept experiments going for several hours without signs of coloration of the reductant.

Solutions.—The solutions were freshly made on the day used, and in the case of unstable compounds like methylene blue they were prepared within a few minutes before use. The indicators were made up in 1 per cent strengths in cold water, filtered if necessary, and the solutions were well stoppered and protected from light and heat. A few of the basic compounds were insoluble in water and to these was added the calculated amount of HCl to produce the chloride. The solubility of some of the substances is low, leucomethylene blue, for instance, is very slightly soluble. Therefore the 1 per cent strength represents merely the maximum possible calculated concentration. Unless otherwise stated, an aqueous solution of the sodium salt of each acid indicator was employed.

Amounts Injected.—The terms small, moderate and large are frequently used here when referring to the volume of reagent injected (*cf.* Chambers and Reznikoff, 1926). The amount was estimated in comparison to the volume of the nucleus or of the ameba. A small amount corresponds approximately to the volume of the nucleus; a moderate amount equals a quarter to a third, and a large amount, one-half the volume or more of the whole ameba.

Dimensions vary a good deal, but if we assume for orientation a spherical shape of 0.2 mm. diameter for the average ameba and 0.01 mm. for its nucleus, the corresponding volumes would then be 0.004 c. mm. and 0.000,000,5 c. mm. Therefore, a "small" injection would be of the order of magnitude of 5×10^{-7} c. mm., a "moderate" injection, 1×10^{-3} c. mm. and a "large" injection, 2×10^{-3} c. mm.

Microscopic Equipment.—Most of the injections were performed under a Leitz No. 5 or a Zeiss No. 20 objective and a 10× or 15× ocular. The No. 3 Leitz double demonstration eyepiece was also employed; in practically all the experiments, the observations recorded were seen by at least two observers; the experiments were repeated until the observers agreed upon the findings. The illumination was the same as that employed and described by Chambers and Pollack (1927).

EXPERIMENTAL RESULTS.

In view of the interpretations involved, it is considered necessary to report certain details of the observations. These will be described as briefly as possible. The essential results are summarized in Table II.

The injections were made mostly within the first 2 hours after the amebæ had been transferred from the culture to the cover-slip. Injections under anaerobiosis were not done until after a preliminary deaeration of the moist chamber for 15 to 30 minutes.

A1. Phenol m-Sulfonate Indo-2,6-Dibromophenol.—The oxidant was injected aerobically. The greenish blue solution decolorized instantly, the injected region was then pinched off, breaking within 2 or 3 minutes. The ameba often survived 5 or 10 minutes before breaking up. With rather large injections, the pinched off

portion remained blue for 1 minute, fading slightly or completely before bursting within 3 minutes. The compound was toxic.

A2. Phenol o-Sulfonate Indo-2,6-Dibromophenol.—The blue oxidant was injected aerobically. In the cytoplasm, the dye decolorized within 10 seconds; it took 30 to 60 seconds to disappear from the nucleus when the latter was stained by the injection. The compound was toxic on injection. Amebæ immersed in the diluted dye remained alive as long as 3 days, without showing internal coloration.

B. Phenol m-Sulfonate Indophenol.—The oxidant was injected anaerobically. The greenish blue solution faded instantly in the cytoplasm, the injected region then pinched off and the ameba broke. The compound was unpurified and pronouncedly toxic.

C. m-Bromophenol Indophenol.—On injecting the oxidant aerobically, the blue solution from the pipet as it entered the ameba showed a pink flash followed by instantaneous decoloration. The ameba recovered.

D. o-Chlorophenol Indophenol.—The oxidant was injected aerobically. The deep blue solution from the pipet on entering the ameba showed an evanescent, purplish pink flash of color which instantly faded. The compound was apparently non-toxic.

E. o-Bromophenol Indophenol.—The pink oxidant was injected aerobically. The dye on entering the ameba assumed a faint pink which faded rapidly.

F. Phenol Blue.—The dye base was treated with an equivalent amount of HCl to produce the soluble chloride. The purple oxidant was injected aerobically and the color faded in the ameba within 3 seconds. The compound is non-toxic; one ameba was injected 5 times without apparent injury. As an indicator it appears unsatisfactory because of instability.

G. Bindschedler's Green.—The zinc chloride salt of the oxidant was injected aerobically and was found to be extremely toxic. The ameba lost all activity and appeared injured. The injected dye did not fade in 3 to 5 minutes, and appeared to accumulate on the nucleus and the granules in the cytoplasm. The oxidant is unsatisfactory also because of its low tinctorial power.

The reductant (free base) was dissolved anaerobically in an equivalent amount of HCl and several amebæ were immersed in the drop anaerobically. The amebæ survived the immersion but showed no signs of coloration.

H. Phenol Indo-2,6-Dichlorophenol.—The blue oxidant injected aerobically or anaerobically into the ameba faded rapidly. The decoloration was so rapid that the color faded at the site of injection before the oxidant could diffuse through the whole organism. The compound is non-toxic, for the amebæ recovered quickly from the injections.

Potassium ferricyanide injected shortly after decoloration of the dye brought back the blue color; when injected 1 hour after decoloration the color was not restored.

I. Phenol Indo-2,6-Dibromophenol.—The oxidant was injected aerobically and the blue color faded from the cytoplasm in about 5 seconds. The fading was

somewhat slower from the nucleus (about 10 seconds). The compound gave evidences of toxicity; but this was an unpurified preparation.

J. m-Cresol Indophenol.—The red oxidant was injected aerobically and decolorized rapidly. The compound is non-toxic.

K. o-Cresol Indophenol.—The red oxidant was injected aerobically and the color faded within 5 seconds. The compound is non-toxic.

L. o-Cresol Indo-2,6-Dichlorophenol.—The oxidant was injected aerobically and anaerobically. In both instances the blue color diffused rapidly through the cell, faded within 5 seconds and the ameba became very active. The compound is not very toxic except for a tendency to disrupt the surface of the cell.

Ferricyanide injected after decoloration of the indicator gave a pronounced return of color.

M. 1-Naphthol-2-Sulfonate Indophenol m-Sulfonic Acid.—This was an impure specimen contaminated with almost 50 per cent NaCl. The red oxidant injected aerobically in small and medium amounts in an active ameba faded within 10 seconds. If injected into a quiescent ameba or if in large amounts, the color persisted. The compound was toxic, and the injected portion was pinched off, as a rule.

The oxidant injected anaerobically decolorized within a few seconds (up to 45 seconds).

Injection of ferricyanide restored the color.

N. m-Toluylene Diamine Indophenol.—This amphoteric compound is practically insoluble in water, and an equivalent amount of HCl was added to produce the soluble chloride. The brown-red oxidant injected aerobically or anaerobically decolorized in 5 to 15 seconds: fading from the nucleus was a little slower. Injection of potassium ferricyanide or chromate restored the brown-red tint.

Although the nucleus and cytoplasm became colorless shortly after injection of the dye, certain inclusion bodies gradually took on a distinct brown-red which persisted at least 24 hours in the active ameba. Brief immersion in a solution of the oxidant caused certain granules in the ameba to take on the color, which faded shortly and which was restored on the granules by the injection of chromate.

The colorless reductant injected anaerobically remained colorless in the ameba. There was no sign of recoloration.

Both the oxidant and reductant are non-toxic, the amebæ recovering completely 1 minute after an injection.

O. 1-Naphthol-2-Sulfonate Indophenol.—The red oxidant was injected aerobically and the intensity of color immediately diminished. A distinct pink tinge persisted for 5 seconds up to 1 minute, according to varying amounts injected, and eventually decolorized. In small amounts the dye was immediately decolorized. The compound is largely non-toxic. In those cases where the color persisted the ameba rounded up, its granules sank, the nucleus took on the color and the organism never recovered.

P. 1-Naphthol-2-Sulfonate Indo-2,6-Dichlorophenol.—This sample was contaminated with about 30 per cent sodium chloride, and exhibited toxic effects.

The oxidant injected aerobically changed to a paler blue and faded completely from the cytoplasm in 1 to 3 (up to 10) seconds, and from the nucleus in 3 to 5 (up to 30) seconds. The nucleus stained a deeper blue than the cytoplasm. With small injections no color remained after 30 seconds; with larger injections, the color persisted for 1 to 2 minutes. On injection of large amounts, the color often remained localized, and the injected area was pinched off. With small injections there was no pinching off and the color disappeared within 5 seconds.

Injection of the oxidant anaerobically caused a localized blue which faded from the cytoplasm within 10 seconds and from the nucleus within 2 minutes. In the active ameba the reduction was rapid; but in the quiescent cell the color persisted.

The colorless reductant was injected under the usual strict anaerobiosis. Many of the amebæ broke while exhibiting a strong sodium ion effect but there occurred no sign of coloration. The injection of partly colored reductant was followed by an immediate fading of the color, which could be restored by ferricyanide. This restored color, which faded again shortly, was most pronounced on the nucleus.

The reductant was toxic and tended to fluidify the interior of the ameba and to cause its surface to break (Chambers and Reznikoff, 1926).

Q. Toluylene Blue Chloride.—The oxidant was injected aerobically. The purple solution from the pipet, on entering the ameba localized at the site of injection as a brilliant purple coagulum before diffusing throughout the cell. The color changed to blue in 10 to 15 seconds and, except for some scattered granules, faded in 30 seconds. The nucleus turned blue and faded in 40 seconds. Some blue and purple granules persisted; and a blue tinge often persisted in the hyaline cytoplasm for many minutes. The amebæ recovered.

Injection of potassium chromate accentuated the blue on the granules and slightly in the cytoplasm.

The oxidant, on injection anaerobically, caused a local solidification which was sometimes extruded; and some of the dye accumulated on the granules. When the injected region was not extruded and the cell was sluggish, the region remained stained for a long time. The active cell decolorized the dye rapidly. When injected on the surface of the ameba, the color penetrated the cell wall. Exposure of the ameba to air or oxygen caused a temporary return of the purple color of the oxidant. The compound appeared to be somewhat toxic and acted on the ameba like methylene blue.

R. Methylene Blue Chloride.—The oxidant injected aerobically usually produced a local blue coagulum; and the nucleus took on an intense blue, while the contractile vacuole remained uncolored. After 1 minute, the cytoplasm became a very pale blue, which sometimes persisted. Occasionally, the stained region was pinched off. Following the local solidification there ocurred some diffusion of the color. The blue coagulum dispersed in fragments most of which decolorized in about 2½ minutes. With time, some color accumulated on certain granules in the almost decolorized cytoplasm. A very small injection of the 1 per cent solution decolorized within 1 minute. Injection of ferricyanide brought back the color especially on the granules.

Injection of the oxidant anaerobically produced a localized blue coagulum and the ameba became quiescent. In the active portion of the ameba, the dye faded quickly (within 1 minute); in the coagulated portion the decoloration took 3 to 4 minutes. In one case, injection of oxidant anaerobically caused the nucleus to take on a green color which began to fade in 10 minutes and decolorized completely in 14 minutes.

Injection of ferricyanide or exposure of the amebæ to air restored some of the color. After transfer from nitrogen to air, the ameba became more active and assumed a pale blue, after which the color accumulated on certain granules and the cytoplasm became colorless.

The oxidant injected anaerobically into *dead* amebæ remained blue indefinitely.

The *reductant* was a saturated aqueous solution of the chloride. It was troublesome to manipulate because it crystallized out and clogged the pipet; and also because it is light-sensitive. The reductant on anaerobic injection produced a local, dark blotch which, upon inspection, proved to be a mass of crystals (apparently leucomethylene blue). There was no local coagulation. The amebæ remained colorless, and readily recovered from the injection. Injection of colorless reductant always left the ameba uncolored. Subsequent injection of ferricyanide or exposure of the ameba to air restored the color of the oxidant temporarily; and the nucleus appeared distinctly bluer than the cytoplasm.

S. K_4 Indigo Tetrasulfonate.—The oxidant is toxic. Injection of the purple oxidant aerobically in very small amounts was followed by a quick disappearance of the color. In large amounts, the ameba colored distinctly blue, which persisted for an hour with only slight fading. The color was localized at the moment of injection and then spread quickly giving the cytoplasm and nucleus a distinct pale blue tinge. In some cases, the blue tinge, before spreading, localized in a large blister-like elevation which became incorporated within 1 to 2 minutes. After 6 minutes the color had paled appreciably especially in the nucleus which seemed to lose the color completely. The contractile vacuole remained uncolored at all times. Injection of ferricyanide brought back no increase of color.

Injection of the oxidant anaerobically caused quiesence; the dye diffused rapidly and stained the nucleus. The color disappeared in from 1 to 7 minutes. With very small injections the color disappeared immediately. Subsequent injection of ferricyanide or chromate or exposure to air restored the purple color in the ameba.

Injection of the reductant anaerobically caused quiescence followed by active movements. The yellow reductant remained yellow in the ameba provided air was excluded. On exposure to air the ameba turned purple; and injection of ferricyanide also restored the purple color. The reductant appeared non-toxic in contrast to the oxidant.

T. K_3 Indigo Trisulfonate.—The blue oxidant is toxic and the injected amebæ exhibited a marked potassium effect. When injected aerobically it imparted a blue color which persisted.

The oxidant injected anaerobically decolorized in the ameba within 4 to 7 minutes ($2\frac{1}{2}$ to 15 minutes in extreme cases). Injection of ferricyanide or exposure to air restored the color.

The reductant injected under strict anaerobiosis imparted only a yellow color to the ameba and a slightly greenish tinge to the nucleus. Partly oxidized reductant injected into the ameba produced a blue color which was quickly reduced. The reductant appeared to be toxic in moderate amounts. Injection of ferricyanide or exposure to air restored the color in the cytoplasm which also exhibited blue granules not previously visible.

U. K_2 Indigo Disulfonate, Also the Na_2 Salt (Indigo Carmine).—The oxidant is very toxic and seemed also to toughen the pellicle. The blue oxidant injected aerobically was not reduced as long as the amebæ were kept under observation (maximum time 45 minutes). On injection, the dye solution did not appear to diffuse through the cell, but rather seeped through channels in the cytoplasm. When the nucleus was near the site of injection it took on a deep blue color. When the nucleus was beyond the site of injection it gradually assumed a blue color while still surrounded by colorless cytoplasm.

The oxidant injected *anaerobically* showed evidences of toxicity and usually caused pinching off. The injection fluidified the ameba and produced a blue cytoplasm and a deeper blue nucleus. In some cases (of apparently injured amebæ) the color was partly reduced, but not completely. When the membrane of the ameba broke, the nucleus lost its intense color and assumed the same tint as the cytoplasm. However, when the ameba survived, the color was gone in 3 to 4 minutes. Injection of ferricyanide restored the blue color which faded again under anaerobiosis. Exposure of the cells to air also restored the color. In air the restored color remained for about 15 to 20 minutes and then seemed to fade as the ameba became more active.

The reductant injected under strict anaerobiosis imparted a greenish yellow tinge to the ameba but no signs of blue. Exposure to air caused the injected cell to assume a deep blue; and injection of ferricyanide also restored the blue color.

V. Neutral Red Iodide.—This basic dye does not seem to possess a labile oxidation-reduction equilibrium at pH 7.0.

The oxidant injected aerobically tends to produce a localized coagulum which stains deep red. With concentrations more dilute than 1 per cent, the color diffused through the cytoplasm giving it a reddish tint with no visible sign of coagulation. When the nucleus was in the immediate vicinity of the injected region, it took on a red color. If the color persisted in the nucleus, the ameba eventually either died or expelled the nucleus by a pinching-off process. The diffuse coloration of the cytoplasm lasts only a few seconds after which the color accumulates on or in granules and vacuoles.

W. Dimethylaminomethylphenazine Chloride.—This compound differs from neutral red by the lack of one amino group.

The oxidant injected anaerobically produced a flash of brilliant orange-red which disappeared at once and left a shower of crystals of the dye inside the ameba. Some of the spear-like crystals stuck out of the ameba. The cell responded by attempting to pinch off the injected portion. The oxidant was toxic. In one case, after pinching off the injected region, the ameba recovered with a pink coloration persisting.

The yellow solution of reductant remained unoxidized in air. Injection of the reductant aerobically and anaerobically colored the ameba yellow, and the color persisted unchanged. The reductant was non-toxic. It did not behave like a basic dye, did not stain the nucleus nor did it coagulate the cytoplasm. The *injected yellow compound tended to accumulate in particles, leaving the background a paler yellow.*

Subsequent injections of ferricyanide did not color the ameba but merely caused the nucleus to clear up and the granules to sink.

X. Phenosafranin.—The oxidant was very toxic and accumulated in the nucleus. Injected anaerobically, it produced a localized coagulum from which the red color diffused into the rest of the cell. The nucleus was intensely stained and the color persisted. In one case, a small amount was injected and the color seemed to disappear within 2 minutes. There was a pronounced staining of the amebæ coming into contact with escaped oxidant in the hanging drop.

The yellow reductant solution is relatively stable in air at pH 7.0; and when injected it imparted a yellow coloration to the ameba. The reductant was relatively non-toxic, did not produce coagulation in the cell and did not accumulate in the nucleus. When the injected ameba was exposed to air, the granules in the cell took on a pinkish tinge. Injection of ferricyanide reddened the ameba which had previously received a moderate injection of reductant; in 14 minutes the granules became salmon-colored, and in 30 minutes they were a distinct pink. In one case, injection of ferricyanide resulted, within 2 minutes, in the appearance of a diffuse pink in the ameba.

RÉSUMÉ OF EXPERIMENTAL RESULTS.

Table II gives the results in summary. The main aspects of the experimental findings may be briefly stated as follows:

1. Under anaerobiosis, *A. dubia* was able to reduce completely all the *reversible* oxidation-reduction indicators tried.

2. Under anaerobiosis, the ameba was unable to reoxidize six of the most easily oxidizable indicators in the list.

3. Under aerobiosis, the ameba was able to reduce completely all but one (a very toxic member) of the reversible indicators from *A1* to *P* (*i.e.*, down to and including 1-naphthol-2-sulfonate indo-2, 6-dichlorophenol). Compounds *Q* and *R* were on the border-line, the reduction under aerobiosis being sometimes complete and sometimes only partial. Compound *S* was only slightly reduced, if at all, while *T* and *U* remained apparently unreduced. The irreversible indicators *V*, *W* and *X* showed questionable reduction.

4. The time of reduction varied approximately with the amount of injection.

5. In general, the speed of reduction was greater under anaerobiosis than under aerobiosis; and there is a suggestion that reduction was more rapid in the active ameba than in one which remained quiescent after the injection.

6. Details of the *speed* of color disappearance should be noted. With the average injection under aerobiosis complete fading was instantaneous or of the order of 5 seconds for all of the indicators from *A1* to *L* (excepting toxic *G*). It required about 10 to 30 seconds for *M*, *N*, *O* and *P*; and it took up to 10 minutes or more to accomplish partial fading of *Q*, *R* and *S*.

7. Toxic compounds as a class were reduced somewhat more slowly than adjacent non-toxic compounds on the rH scale.

8. Toxicity varied considerably, owing partly to inaccurate control of dosage and to impurities. Compound *G* (zinc chloride salt of Bindschedler's green) was immediately fatal. The simple indophenols were generally non-toxic. The sulfonated compounds as a class were toxic to different degrees. The reduced compounds were usually less toxic than the oxidants.

TABLE II.

Results of Injections of Oxidation-Reduction Indicators into the Cytoplasm of Amœba dubia.

Indicator	Aerobic injection	Anaerobic injection		Toxicity
	Oxidant	Oxidant	Reductant	
A1	Reduced	—	—	Toxic
A2	Reduced	—	—	Toxic
B	—	Reduced	—	Very toxic
C	Reduced	—	—	Non-toxic
D	Reduced	—	—	Non-toxic
E	Reduced	—	—	—
F	Reduced	—	—	Non-toxic
G	Not reduced*	—	—	Extremely toxic
H	Reduced	Reduced	—	Non-toxic
I	Reduced	—	—	Toxic
J	Reduced	—	—	Non-toxic
K	Reduced	—	—	Non-toxic
L	Reduced	Reduced	—	Slightly toxic
M	Reduced	Reduced	—	Toxic
N	Reduced	Reduced	Not oxidized	Non-toxic
O	Reduced	—	—	Slightly toxic
P	Reduced	Reduced	Not oxidized	Toxic
Q	Reduced partly	Reduced	—	Slightly toxic
R	Reduced partly	Reduced	Not oxidized	Slightly toxic
S	Slightly or not reduced	Reduced	Not oxidized	Oxidant toxic; reductant non-toxic
T	Not reduced	Reduced	Not oxidized	Oxidant toxic; reductant slightly toxic
U	Not reduced	Reduced	Not oxidized	Toxic
V	Not reduced	—	—	—
W	Not reduced	Not reduced?	Not oxidized	Oxidant toxic; reductant non-toxic
X	Not reduced	Slightly reduced?	Not oxidized	Oxidant toxic; reductant non-toxic

* In moribund or dead cells; zinc salt of dye used.

Sources of Error.

Before proceeding to an interpretation of the experimental results, it is necessary to consider a few of the sources of error.

Critique of Microinjection Method.—With the recent refinements in construction of the apparatus, the manipulations incident to micro

injection can be performed with ease, and furnish an elegant method of attack on various problems in cellular biology. For direct observation of certain qualitative changes, the method is quite unexcelled in its delicacy and the definiteness of approach. As will be seen from the experiments and in the discussion to follow, certain quantitative aspects of the problem became of paramount importance for a proper interpretation of the results. The mere observation that a dye is reduced within the cell is clearly only a first step; when we come to compare the reduction of different dyes and note differences in the *speeds* of reduction, we face at once the need for strict quantitative comparisons. This is where the microinjection technic in its present stage of development fails to satisfy except in a very gross way. It is as yet impossible to gage precisely, as ordinarily understood in quantitative work, the volume of solution injected intracellularly.

One must not leave unmentioned in this connection the microscopic equipment and the illumination, nor yet the personal factor. The observations were primarily those of color change and fading in the microscopic field. Obviously the lens system must be of a type which will reduce intrinsic coloration to a minimum; and more important still, the source of illumination and screening should be satisfactory from the same standpoint. Not the least important is the condition of the observer's eye and his acuity of color perception. Prolonged and excessive observation through the microscope, even under almost ideal conditions, induces eye strain and fatigue which definitely inhibit quick and accurate color perception, especially when one must decide if a color is entirely faded. The observer must also be on his guard to avoid misinterpretations of color due to artefacts such as those caused by varying depth of the drop, shadows cast by the micro needles and colors due to natural pigment or inclusion bodies in the cell under observation.

Possible Injury to Amebæ.—In considering our results, one may ask to what extent the observations were affected by possible injury to the organisms studied. A full discussion of the question would take us too far afield. To assert that the manipulation of the cells left them in "normal" condition would depend largely on the definition of the term normal.

The amebæ remain alive for at least 24 hours in a hanging drop in

the moist chamber with free access of air. At the end of that period, the cells appear to be quite active and show no *visible* signs of abnormality. As for amebæ injected with water, the observations of Howland and Pollack (1927) show that the cells usually recover quite completely from the injury incident to injection.

One may urge that the ameba cannot be "normal" after suffering injury from the injection needle. This criticism may perhaps be valid if applied to the case of highly differentiated cells; but we believe that it can apply, if at all, only to a very limited degree to *A. dubia* and then only for a relatively short period immediately following the injection. The ameba exhibits very remarkable powers of readjustment and recovery. Pricking increases the internal flowing movements and there is a quick renewal of the plasmalemma, following which the cell continues to subsist without visible signs of permanent injury.

The situation is somewhat different when the amebæ are kept for short periods in an atmosphere devoid of oxygen. Instead of the typical stellate shape spread out on the under side of the cover-glass, the organism after an exposure of about 30 to 50 minutes assumes a *Limax* form and no new, extended pseudopodia are evident such as are to be seen in the resting stage. Occasionally, there may be seen an ameba with 10 to 15 short lobate pseudopodia. However, except for these changes the cells give no evidence of injury. We have maintained them for as long as 9 to 10 hours under anaerobiosis and then on exposure to air, the cells became more active, lost their *Limax* form and seemed none the worse for the experience.

The introduction of foreign material by gross assault on the cell is no doubt drastic treatment. However, with respect to most of the compounds which we have injected, this criticism loses its force because a number of the indicators (some of which can be taken up by staining) are vital dyes and the cells appear to tolerate them in their interior indefinitely. Moreover, toxic quantities of the compounds simply kill the cells or render them moribund, and our observations and conclusions are based mainly on the response of cells which have survived the operation.

It is easily demonstrable that the injection of a suitable dye into a moribund ameba or into the dead dêbris is followed

reduction of the dye. If the color disappears, the dye is obviously washed out. On the other hand, injection of the dye into a *living* cell is followed by a fading of color which can be restored with a ferricyanide injection. The important fact is that the living ameba exhibits a reducing intensity and capacity equivalent to its ability to perform the work necessary for the intracellular reduction of appropriate compounds. That is, a cell capable of causing the reduction is, if not normal, at least living and still capable of performing work.

Toxicity of the Indicators.— Due to the inherent limitations in the technic the doses injected varied probably very widely. In addition, some of our compounds were known to be more or less contaminated. Another variable factor was the general condition of the different lots of amebæ used in the various experiments. However, with obvious reservations, certain conclusions as to the toxicity of the injected indicators may be made.

Compound *G* was almost instantly fatal, undoubtedly because of the zinc chloride present. There is no theoretical reason for believing that the free base is any more toxic than methylene blue. The simple indophenols as a class were non-toxic; and when contaminated with much salt they produced typical sodium ion effects (Chambers and Reznikoff, 1926).

The different basic indicators exhibited different degrees of toxicity. The amphoteric amino-indophenol *N* was non-toxic. Methylene blue and toluylene blue were slightly toxic. The diazines *W* and *X* were decidedly toxic; in this connection, mention should be made of Marston's (1923) observation that diazines precipitate proteolytic enzymes.

We observed also that toxicity on injection is not necessarily paralleled by toxicity on immersion in the indicator. Attention is drawn to the interesting fact that the reductants of toxic oxidants were usually non-toxic. The sulfonated compounds as a class were more or less toxic; but so far as could be observed, there was no parallelism between the degree of toxicity and the number of sulfonate groups in the compounds studied.

Effect of Sulfonic Acid Radicals on Reduction.—Dixon (1926) found in the case of xanthine oxidase, which he believes is the type of system

responsible in large measure for the reducing power of all living cells, that the velocity of reduction of certain of the oxidation-reduction indicators was appreciably retarded by those indicators possessing sulfonic acid radicals in their structure. Consequently, he suggests, the apparent reductions of such indicators within the cell may depend not so much on the intrinsic reducing power of the cell as upon whether the indicator is sulfonated or non-sulfonated.

In this connection our observations may be of interest. There were nine compounds in the present series which contained one or more sulfonic acid radicals (compounds *A1*, *A2*, *B*, *M*, *O*, *P*, *S*, *T*, *U*). Indicators *A1*, *A2* and *B* were on the most easily reducible end of the series and although definitely toxic, they were reduced in the ameba with considerable rapidity. Their rates of reduction however appeared to be slower than those of adjacent non-sulfonated dyes.

Compounds *M*, *O* and *P* were in the middle range of the series, approximately in the zone where the Needhams observed the critical reduction level of *Amœba proteus*. *M* has two sulfonic acid radicals, and *O* and *P* contain one each. *M* and *P* were toxic, but they were heavily contaminated with NaCl; while *O* was only slightly, if at all toxic. These dyes were completely reduced in the surviving cells within a minute or two.

Compounds *S*, *T* and *U* (indigos) contained 4, 3 and 2 sulfonic groups respectively, and they all exhibited marked toxicity. Under aerobiosis only *S*, the most heavily sulfonated compound, gave evidences of a slight reducibility; but under anaerobiosis, all three were reduced in 5 to 7 minutes (maximum, 15 minutes).

Judged by their speeds of reduction, the sulfonated indicators behaved very much like the non-sulfonated ones, *e.g.*, those on the positive end of the scale decolorized in a few seconds, those in the middle in a minute or two and those on the negative end in 5 to 15 minutes. A slight retardation in the reduction of the sulfonates was noted, but it was of a much lower order of magnitude than that reported by Dixon for the xanthine-oxidase system.

Therefore, despite a certain degree of toxicity which undoubtedly influences cellular processes, the sulfonated indicators were not found to yield a false index of the gross reducing *intensity* in the surviving ameba.

DISCUSSION AND CONCLUSIONS.

We may now consider the results of our experiments as a whole and seek an interpretation of the facts. In this connection, the review by Needham and Needham (1926) and the paper by Cannan, Cohen and Clark (1926) are of interest. Three major factors may be discerned in the reduction processes under our experimental conditions. These are the *intensity* factor, the *capacity* factor, and the *time* or *rate* factor. They have been ignored, confused or inadequately considered in certain recent papers on the subject, which cannot be discussed here. The importance of the matter, however, merits more careful and critical consideration.

Reduction Intensity.—As regards the intensity or potential factor of reduction inside the cell of *Amœba dubia*, the following facts seem clear. The living ameba generates a reduction potential which is sufficiently high to condition, under anaerobic environment, the complete reduction of all the reversible oxidation-reduction indicators down to and including indigo disulfonate. The latter when 99 per cent reduced corresponds to rH 7.5 at pH 7.0, or rH 7.6 at pH 6.9.

The presence of a high intracellular reducing intensity is corroborated by the failure to secure under anaerobiosis even partial reoxidation of six of the most easily oxidizable compounds (namely, *N*, *P*, *R*, *S*, *T* and *U*).

In the aerobic ameba, we find a reducing intensity ranging from rH 13 to 18; the Needhams report for the same species the narrow zone, rH 17 to 19. When there is present the neutralizing effect of atmospheric oxygen, the *apparent* level of intracellular reducing potential rises to a broad zone covered by as many as three indicators, *viz.:* toluylene blue, methylene blue and indigo tetrasulfonate, depending on the capacity and rate factors as discussed below. The particular level found appears to vary with the amount of indicator injected and the duration of observation. One may therefore question if any restricted *portion* of this range represents a unique physiological attribute of the ameba. In general, we find in the aerobic ameba the well known effect found in aerated suspensions of cells and "metabolites," namely, a gross reducing intensity stabilized between 0.1 and 0.2 volts at pH 7. The probable physiological significance of this has

already been discussed by Needham and Needham, and by Cannan, Cohen and Clark (1926).

Our results show that the series of satisfactory indicators is incomplete, and must be extended down to and beyond the hydrogen electrode potential before a final conclusion can be drawn, from this type of experiment, as to the limiting reduction potential attainable inside the ameba. In this connection, nothing satisfactory can be elicited from the behavior of the three irreversible compounds in our series.

This much however may be stated with confidence. The ameba develops in its interior a high, primary, reducing potential with a value certainly less than rH 7.6. In the presence of the neutralizing effect of atmospheric oxygen, the internal rH appears to assume any value between 13 and 18 depending on secondary factors.

If the rate of oxygen activation in the cell or at the cell surface is a function of the pressure, we can predict that the apparent internal rH will depend also on the oxygen pressure; it should certainly do so at very low oxygen tensions.

Reducing Capacity.—The quantity of material that the ameba can reduce will depend primarily on the reduction potential it can generate, and secondarily on the quantity of reducing substance present or generated. The evidence offered by these experiments on the capacity factor is only indirect because the use of accurate quantities of solutions was precluded. We found it possible to swamp out the reducing capacity of an ameba by five successive injections of a non-toxic, easily reducible compound. The result was a living ameba colored for several minutes by an indicator representing a high, "unnatural" rH.

Another aspect of the capacity factor appears in the case of certain basic dyes which tend to accumulate upon granules in the cytoplasm. This matter deserves further study.

If the speed of reduction is a criterion, it seems that even under aerobiosis the ameba ordinarily has available a quantity of active reducing material sufficient to completely reduce moderate injections of 1 per cent solutions of the various simple indophenols (compounds *A* to *M*). The behavior of the rest of the reversible indicators is more involved but, making allowance for factors of toxicity and granular accumulations, etc., there appears to exist

as if material of high reducing potential were not immediately available in sufficient quantity.

The fact that under anaerobiosis even small injections of completely reduced indicators remained completely reduced within the ameba, and that partly reduced compounds were quickly decolorized points quite clearly to an important conclusion: namely, there was not available an appreciable quantity of cellular oxidation-reduction substances poised at any rH point covered by the indicators employed. Such a level may exist, but for the particular organisms studied, namely, *A. dubia* and *A. proteus*, it lies below the range of indigo disulfonate and in the direction of the hydrogen electrode level.

How does this absence of poising ability in the rH range investigated harmonize with our finding that even under aerobiosis the ameba has available an appreciable quantity of active reducing material? In this connection one must remember that poising ability (the capacity to maintain a particular reduction potential level) depends on the presence of finite quantities of both oxidant and reductant in equilibrium with each other. Consequently, the apparent absence of poising ability and the failure to oxidize reduced dyes indicates that, in the rH range studied, the anaerobic ameba contains *only active reductants* in appreciable amount. (This refers, of course, only to the over-all effect in the cell, and may not apply to localizations.) Interference of oxygen complicates the picture by introducing oxidants of all shades of activity and elaborated presumably at varying rates.

The Rate Factor.—On this point also, our observations are necessarily indirect. We have already noted the retardation of reduction by toxic compounds. Our observations suggest that a kinetic study of intracellular reduction is experimentally feasible and of important promise.

It is of course precarious to apply to the dynamic activity of the heterogeneous system of the cell some of the criteria established for the essentially static, homogeneous indicator systems. In the latter the postulates of complete, labile reversibility and practically instantaneous equilibrium are important criteria. To apply these to the cell system requires the introduction of the time factor and possibly a host of phase and phase boundary equilibrium factors which are as yet only vaguely understood.

Despite the complexity of the subject, it is not difficult to discern a sequence of events which is reproducible, and which seems logically attributable to the high reducing intensity of the living cell interior, to the quantity factor and the time factor.

The almost instantaneous reduction of the indicators on the electropositive side is strong evidence for the existence of a virtual labile, reversible equilibrium state. The slower reduction rate of indicators on the electronegative side may merely indicate an inadequate capacity factor rather than any essential difference in the degree of lability of the equilibrium.

These considerations necessarily assign to molecular oxygen a secondary, neutralizing rôle dependent on its rate of diffusion and activation. This secondary effect may become appreciable as soon as its magnitude equals or exceeds the net biological effect. From the work of Brooks (1926), Rapkine and Wurmser (1926) and Cannan (1926) on chlorophyll-bearing cells, it is now known that relatively high reducing potentials can be maintained apparently concurrently with an active production of molecular oxygen by the chloroplasts within the cell. To what extent this phenomenon and the rate factor are influenced by the action of "antioxidants" remains an interesting question to be determined.

The present indicator studies on the ameba show an almost complete parallelism with the reduction electrode studies on bacterial suspensions reported by Cannan, Cohen and Clark, with the very important difference that the center of interest has been shifted from the mass culture to the individual cell. These results stand somewhat in contradiction to those reported by Needham and Needham for *A. proteus*, and render it impossible to accept the validity of a number of their conclusions. However, it is possible that their organism was entirely different from our *A. dubia* and *A. proteus*, since the internal pH of the Needham strain is reported at about 7.6 while Chambers, Pollack and Hiller found for our two strains a pH of about 6.9. Our criteria and technic were also somewhat different from those employed by the Needhams, therefore a critical comparison would be out of place. However, if the strains in Europe and America are comparable, our results indicate that the conclusions of the Needhams with regard to ameba will have to be modified in several important

respects to include the wide rH range under aerobiosis, the high reducing potential under anaerobiosis and the capacity and time factors, as we have shown above.

It is well known that ameba feeds on bacteria and other small organisms, and it is not improbable that certain bacteria may live symbiotically in the interior of the ameba. This possibility has a significant theoretical bearing and it might, perhaps, account for part of the discrepancy of our findings and those of the Needhams. However, direct experimental evidence is needed.

SUMMARY.

Twenty-five oxidation-reduction indicators were injected in oxidized or reduced form into *Amœba dubia* and *Amœba proteus* under controlled conditions of oxygen access. (1) Under anaerobiosis the ameba was able to reduce completely all the reversible oxidation-reduction indicators down to and including indigo disulfonate. (2) Under anaerobiosis the ameba was unable to reoxidize six of the most easily oxidizable indicators. (3) Under aerobiosis the ameba was able to reduce completely all the indicators down to and including 1-naphthol-2-sulfonate indo-2, 6-dichlorophenol. Toluylene blue, methylene blue and indigo tetrasulfonate were sometimes completely and sometimes only partly reduced, depending on the quantity of indicator injected and the duration of observation. (4) The time of reduction varied approximately with the size of the injection. Reduction was more rapid under anaerobiosis than under aerobiosis, more rapid in active than in sluggish cells and was retarded by toxic compounds. (5) Sulfonated compounds were somewhat toxic, as a rule.

In interpreting reduction phenomena of micro injection, it is necessary to take into consideration the intensity, capacity and rate factors. It then becomes apparent that the ameba has a high reducing potential lying on the rH scale below the zone of indigo disulfonate. The reducing capacity of the ameba seems to be relatively great in the region of the simple indophenols and of a progressively diminishing magnitude as the zone of the indigos is approached. Material of high reduction potential appears to be generated within the ameba at a measurable rate. These phenomena, observed in the interior of the

cell with the aid of indicators, parallel very closely those found in reduction electrode studies on bacterial cultures.

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THE IONIC ACTIVITY OF GELATIN.

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I.

INTRODUCTION.

In two other papers[1] it was shown that the monoions of weak acids in dilute solution obey the limiting Debye-Hückel equation:

$$-\log f = a\,\nu^2\sqrt{\mu} = a\,\nu^2\sqrt{\frac{1}{2}\,i\,\nu^2} \tag{1}$$

while the polyvalent ions, in the presence of NaCl, obey a modified equation:

$$-\log f = a\,\nu'\sqrt{\frac{1}{2}\,i\,\nu'} \tag{2a}$$

where

$$\nu' = \nu^2 - (\nu^2 - \nu)\,\frac{r_x}{18} \tag{2b}$$

where r_x is the distance in Ångstrom units between like charges in a polyvalent ion. However, these polyvalent ions show a large deviation in one direction with Mg^{++} ions and a small deviation in the opposite direction with SO_4^{--} ions. Furthermore, the ions of ampholytes obey the above equations in the presence of NaCl but are anomalous with $MgCl_2$. On the other hand, the cations of amines or ampholytes do not obey the Debye-Hückel law either with NaCl or $MgCl_2$.

[1] Simms, H. S., *J. Phys. Chem.*, 1928, pending publication.

The fact that anions of ampholytes obey the modified Debye-Hückel equation (2*a* and *b*) gives us a means for studying a protein. Gelatin was chosen for the purpose.

In the study of simple substances we plotted the square root of the ionic strength ($\sqrt{\mu}$) against values of the uncorrected dissociation indices (pK′ values) for each index of the substance. The slopes of these curves equal Sa, where

$$\pm S = \nu^2 - (\nu - 1)^2 = (2\nu - 1) - (\nu - 1)\frac{r_z}{9} \tag{3}$$

where ν is the valence of the ion produced by a given step in ionization, and $\pm S$ has the sign of the valence. In other words, for a given dissociation index:

$$\text{pK}' - \text{pK} = S\,a\,\sqrt{\mu} \tag{4}$$

where S has the value given by equation (3).

II.

Ionic Activity of Proteins.[2]

The above method cannot be applied to proteins since we cannot obtain pK′ values with any accuracy. However, we may use the following method: Let us consider two solutions of a weak electrolyte in equal concentrations and having the same equivalents of base (b') but differing in their ionic strength (due to the addition of salt to one of them). The difference (ΔpH) between the hydrogen indices of these two solutions will be the same as the difference (ΔpK′) between the uncorrected dissociation indices and will equal Δ(−log f), hence:

$$\Delta\text{pH} = \Delta\text{pK}' = \Delta(-\log f) = S' \cdot a \cdot \Delta\sqrt{\mu} \tag{5}$$

or

$$S' = \frac{2\,\Delta\text{pH}}{\Delta\sqrt{\mu}} \tag{6}$$

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[2] Attempts to determine or correct for the activity of proteins have been made by others, for example, Cohn, *Physiol. Rev.*, 1925, v, 349. Sørensen, Lang, and Lund, *J. Gen. Physiol.*, 1925–28, viii, 543. Stadie and Hawes, *J. Biol. Chem.*, 1927, lxxiv, p. xxxi.

This S' is the correct value of S only if b' is the same in both solutions. If it is different (as will be the case at high or low pH) we must write:

$$S = \frac{\Delta \mathrm{pH} - \Delta b'/\beta'}{a \cdot \Delta\sqrt{\mu}} = S' - \frac{2\,\Delta b'}{\beta' \cdot \Delta\sqrt{\mu}} \tag{7}$$

where $\beta' = \dfrac{\Delta b'}{\Delta \mathrm{pH}}$ is a modification of Van Slyke's "buffer value"[3] and represents the equivalents of base required to produce unit

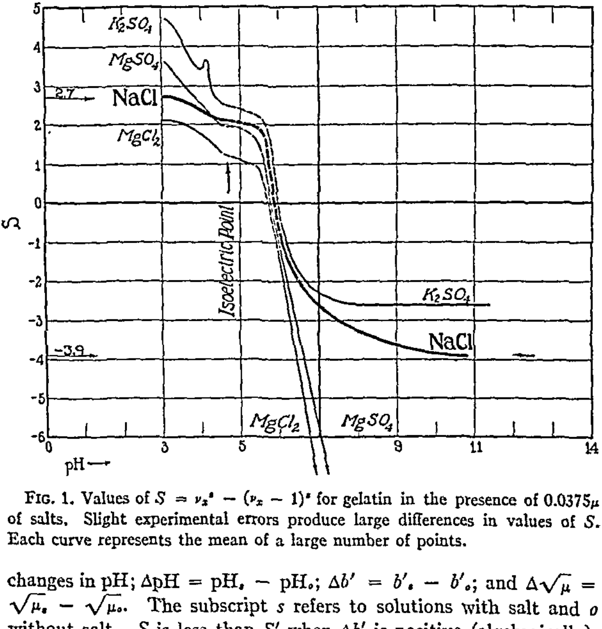

FIG. 1. Values of $S = \nu_x^2 - (\nu_x - 1)^2$ for gelatin in the presence of 0.0375μ of salts. Slight experimental errors produce large differences in values of S. Each curve represents the mean of a large number of points.

changes in pH; $\Delta \mathrm{pH} = \mathrm{pH}_s - \mathrm{pH}_o$; $\Delta b' = b'_s - b'_o$; and $\Delta\sqrt{\mu} = \sqrt{\mu_s} - \sqrt{\mu_o}$. The subscript s refers to solutions with salt and o without salt. S is less than S' when $\Delta b'$ is positive (algebraically).

[3] Van Slyke, *J. Biol. Chem.*, 1922, lii, 525, and Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1249. The β' values used in Table IV are those of the gelatin titration curve without salt.

III.

Results of Study of Gelatin.

We have obtained data on gelatin from pH 3 to 11. At numerous values of $\frac{b-a}{c}$ (equivalents of base) we obtained the pH of solutions without salt and at two different concentrations each of NaCl, $MgCl_2$, K_2SO_4, and $MgSO_4$. The values of ΔpH and of[4] $\Delta\sqrt{\mu}$ were used in equation (6) to calculate S'. The values of S were then calculated from equation (7) and are presented in Table IV and plotted in Fig. 1 for the dilute solutions ($0.0375\,\mu$) of salts. The curves for concentrated solutions ($0.075\,\mu$) are essentially the same but show slight deviations due to the higher ionic strength.

Previous data showed that in the presence of NaCl, anions of weak electrolytes behave normally (and cations show small deviations) while the presence of Mg^{++} ions or $SO_4^{=}$ ions causes large deviations.

[4] The values of $\sqrt{\mu}$ were obtained as follows: The *total* ionic strength is:

$$\mu = \mu_s + \mu_o \tag{8}$$

where μ_s is the ionic strength due to added salt (0.0375 and 0.0750, respectively in the two concentrations) and μ_o is the ionic strength of the solution having no added salt. The latter is calculated as follows: If the "apparent valence" of protein is unity, we may write:

$$\mu_o = \pm\, b'\,c + h + oh \tag{9}$$

(where $\pm b'c$ is always a positive number), but with an "apparent valence" of ν_A we get

$$\mu_o = \pm\,\frac{\nu_A{}^2 + 1}{2}\,b'\,c + h + oh \tag{10}$$

Our preliminary estimation of S_M was 2.0 in acid solution and 3.0 in alkaline solution. Equation (15) gives values of ν_A equal to 1.5 and 2.0. These were used in equations (8) and (10) to obtain the μ values used in Table IV. The final values of S_M are 2.7 and 3.9 (corresponding to ν_A equals 1.85 and 2.45). Although we would expect the latter values would be more correct, they appear to be too high. As an approximation we have used the same value (1.5) of ν_A at all points in the acid titration and the same value (2.5) at all points in alkali titration and we have neglected h and oh in equation (10).

TABLE I.

Deviations (ΔS) Produced in Weak Electrolytes by 0.0375μ of Salts.

Substance	Distance r_x	Valence ν	Apparent valence ν_A	ΔS in acid titration $MgCl_2$	ΔS in alkaline titration $MgCl_2$	ΔS in alkaline titration $MgSO_4$	ΔS in alkaline titration K_2SO_4
	Å						
Citric acid..........	7.4	2	1.7		−9		
Citric acid..........	7.4	3	2.2		−29		
Oxalic acid..........	4.5	2	1.8		−17		
Malonic acid........	5.7	2	1.7		−8.3	−5.0	+0.7
Succinic acid........	7.0	2	1.6		−1.3		
Azelaic acid.........	12.1	2	1.3		−0.8		
Aspartic acid........	7.0	2	1.6		−9.5		
Aspartic acid........		1	1	−0.2			
(Glycine)...........		1	1	−0.2	−6		
(Aminoethanol).....		1	1	−0.8			

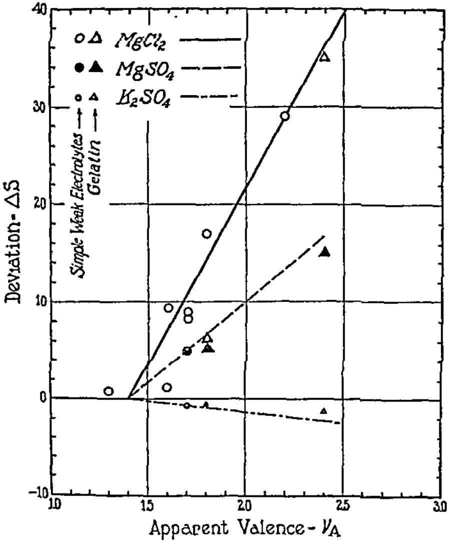

FIG. 2. Relation between "apparent valence" and the deviation proc by 0.0375μ of various salts on simple weak electrolytes and on gelatin. The correspond to equations (12*a*), (12*b*), and (12*c*).

Hence we will take the NaCl data as "normal" and note the deviations caused by other ions. This deviation will be:

$$\Delta S = S_o - S_x \tag{11}$$

where S_o is the value of S with NaCl, and S_x is the value of S with any other salt.

The values of ΔS for some ions of simple weak electrolytes are given in Table I and are plotted in Fig. 2. It will be seen that the data with $MgCl_2$ fall roughly on the empirical curve with the equation

$$\nu_A = 1.4 - \frac{\Delta S}{25} \tag{12a}$$

If we assume the same origin for the curves of $MgSO_4$ and K_2SO_4, and use the data with malonic acid to determine the slope, we get, for $MgSO_4$

$$\nu_A = 1.4 - \frac{\Delta S}{17} \tag{12b}$$

For K_2SO_4:

$$\nu_A = 1.4 + \frac{\Delta S}{2.3} \tag{12c}$$

These equations apply only to the effect of 0.0375μ of these salts on polyanions.

The effect of these salts on gelatin is given in Table II where the values found in alkaline titration (Columns 7 to 9) are seen to agree with those calculated by the above formulas (Columns 10 to 12). These points for gelatin are plotted in Fig. 2 (triangles) and are seen to agree with the curves found for the simple weak electrolytes.

In acid solution gelatin behaves like aspartic acid or glycine with $MgCl_2$. Unfortunately we have no data on polycations with which to make quantitative comparison in acid solution. The qualitative behavior agrees with our expectations.

On the basis of these observations there is every reason to believe that gelatin in dilute solution (2.5 per cent or less) behaves like a polyvalent ampholyte with distant ionizable groups[5] and that the

[5] The nature and sources of these groups will be discussed in the following paper.

ionization is purely that of a weak electrolyte showing the same effects in the presence of Mg^{++} or $SO_4^{=}$ ions that are shown by the simple weak electrolytes.

These deviations are similar to the combination of proteins with inorganic ions as observed by other methods[6] and the combination of simpler substances with inorganic ions.[7]

TABLE II.

Deviations (ΔS) Produced in Gelatin by 0.075μ of Salts (Distance r_x >18 Å.u.)

pH	Valence ν	Apparent valence ν_A	ΔS values: Found in acid titration			Found in alkaline titration			Calculated for alkaline titration		
			$MgCl_2$	$MgSO_4$	K_2SO_4	$MgCl_2$	$MgSO_4$	K_2SO_4	$MgCl_2$	$MgSO_4$	K_2SO_4
3.4	57	1.8	−0.6	+0.2	+1.1						
7.1	16	1.8				−6.3	−5.3	+0.5	−15	−6.5	+0.9
11.0	43	2.4				−30 to −40*	−15*	+1.3	−36	−17	+2.3

* The values for $MgCl_2$ (−30 to −40) and $MgSO_4$ (−15) at pH 11 were estimated by extrapolation.

IV.

Relation between Activity, Valence, and Distance.

Assuming a given molecular weight we may calculate the corresponding number of acid or basic groups from the base or acid "combining capacity." This calculated valence (ν_x) will be a large number

[6] Northrop and Kunitz, *J. Gen. Physiol.*, 1925–26, ix, 351 and unpublished data. Hastings and Sendroy, *J. Biol. Chem.*, 1927, lxxi, 723. Loeb, R. F., and Nichols, *J. Biol. Chem.*, 1927, lxxiv, 645. Adair, *J. Biol. Chem.*, 1925, lxiii, 517, 529. Austin, Sunderman, and Camack, *J. Biol. Chem.*, 1926, lxx, 427.

[7] Pfeiffer and collaborators, *Z. physik. Chem.*, 1924, cxxxiii–cxliii.

In this connection we will mention that a quantitative measurement of the interaction of glycine and phosphoric diion gives the mass action equation:

$$k = \frac{[\text{glycine}] \times [HPO_4^{=}]}{[\text{Combined}]} = 0.040$$

as shown by the effect of glycine on pK'_2 of H_3PO_4. The data will be published later. Similar, but more complex, relations have been found to apply to the effects of Mg^{++} and of $SO_4^{=}$ on ions of oxalic acid.

which if used in the Debye-Hückel equation (1) will give impossible values for the effect of protein on ionic strength. This is because equation (1) assumes the charges to be located at a single point.

A polyvalent ion with charges very far apart would behave like a number of univalent ions. Equation (2) is derived to allow for finite distances between like charges. The mean distance r_x may be calculated from the equation (derived from equation (3)):

$$r_x = \frac{9(2\,\nu - S - 1)}{\nu - 1} \tag{13}$$

From the base-combining capacity (1.75 equivalents per 2500 gm.) and the acid-combining capacity (2.30), we may calculate the maximum valence ν_M of acid and basic groups corresponding to various assumed molecular weights.

TABLE III.

Assumed molecular weight	ν_x No. of acid groups	ν_x No. of basic groups	Mean distance r_x (in Å.u.)	
			Between acid groups ($-S_M = -3.9$)	Between basic groups ($S_M = 2.7$)
96,000	67	88	17.6	17.8
61,500*	43	57	17.4	17.7
30,000	21	27.5	16.7	17.4
20,000	14	18.4	16.0	17.1

*See Foot-note 8.

The NaCl curve in Fig. 1 reached a maximum (S_M) of + 2.7 and a minimum ($-S_M$) of -3.9 (in acid and alkaline solutions, respectively). With these values of ν_M and S_M we may calculate the mean distance r_x from equation (13). These are given in Table III for various assumed molecular weights.[8]

[8] The value 96,000 was obtained by Smith, *J. Am. Chem. Soc.*, 1921, xliii, 1350. The more accurate value of 61,500 (at 25°C.) has since been obtained by Kunitz, *J. Gen. Physiol.*, 1926–27, x, 811. The arbitrary values 30,000 and 20,000 are included in Table III to show that the molecular weight has little effect when the valence is above 10.

Since the formula probably gives low values for long distances, we conclude that the distances are *18Å. u. or over.*

The *probable* distance may be estimated if we consider that the *minimum* distance between acid groups in a protein molecule is about that in aspartyl-aspartic acid anhydride, which is 10 Å.u. Only 1 amino acid molecule in 14 in gelatin is a dicarboxylic acid. Hence the probable mean distance is 30 to 60 Å.u.; but since some will be closer than others the *effective* mean distance will be less, probably between 18 and 25 Å.u.

Another check on this distance is found in the "titration index dispersion."[9] That calculated for 18 Å.u. or over, is 0.7 or less. This agrees with the titration curve which has a dispersion of not more than 0.7, and apparently much less.

V.

The Gelatin Molecule.

The data indicate that the gelatin molecule is large; that the dielectric constant of the medium between these groups is not greatly different from that of water;[10] and that the free ionizable groups are all functioning and are accessible to the inorganic ions in solution. We conclude that the protein molecule is spongy or arborescent in shape with molecules of solvent and of other solutes invading the interstices. It is reasonable to suppose, furthermore, that the shape and size of the molecule changes with pH since the like charges will repel each other[10] and the increase in ionic strength may also influence the

[9] The term "titration index dispersion" is used to refer to the difference between two "titration indices" having the same intrinsic indices. Thus since oxalic acid is symmetrical both groups have the same intrinsic indices (2.55); but its "titration index dispersion" ($pG_2 - pG_1$) is 2.93. In the case of sebacic acid $pG_2 - pG_1 = 0.88$. These values depend upon the distances r_d (4.5 and 14.3, respectively) and the distances r_m (1.2 and 4, respectively) between the charges in the diion and the monoion.

A divalent acid with $r_d = 18$ and $r_m = 5$ would have a dissociation index dispersion of $\Delta pK = 0.85$ or a *titration index dispersion of 0.7*, while a greater distance would give a smaller dispersion.

[10] Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1251.

shape.[11] Such a change in shape and size would be roughly analogous to the opening and closing of a flower.

VI.

The Apparent Valence of Gelatin.

If we assume a point charge (an impossible condition) r_x in equation (3) will equal zero and we get:

$$\pm S = 2\,\nu_A - 1 \tag{14}$$

or

$$\nu_A = \frac{\pm S + 1}{2} \tag{15}$$

where ν_A is the "apparent valence" under the assumption of a point charge ($\pm S$ is always a positive number). If we use the maximum ($S_M = 2.7$) and minimum ($-S_M = -3.9$) values of S obtained from Fig. 1 we find that the "maximum apparent valence" is $\nu_A = 2.4$ for acid groups (in alkaline solution) and $\nu_A = 1.8$ for basic groups (in acid solution). These apparent valences have no physical significance but we may substitute ν_A^2 in the unmodified equation (1) to obtain the *maximum* effect of gelatin on the ionic strength.[4] The *true* effect appears to be even lower than the above values would indicate.

VII.

EXPERIMENTAL.

The gelatin used was ash-free isoelectric gelatin kindly furnished by Dr. John H. Northrop.

Most of the data were obtained on solutions which were 0.005 M per 2500 gm. (an arbitrary molecular weight), namely 1.25 per cent gelatin. The solutions at high and low pH were twice as strong

[11] The last two conclusions are contrary to Svedberg and Nichols (Svedberg and Nichols, *J. Am. Chem. Soc.*, 1927, xlix, 2920) whose primary assumptions were that the hemoglobin molecule is spherical and its size is independent of pH. See also Ghosh, *J. Chem. Soc.*, 1928, cxxxiii, 117.

TABLE IV.

Titration Data of Gelatin without Salt and in the Presence of 0.0375μ (D.—) and and 0.0750μ (C.—) of Salts.

In order to condense this table we give only about half the experimental data used in Fig. 1.

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	1.808	−4.000*			
None	2.133	−3.000*			
None	2.448	−2.500*			
None	2.894	−2.000*			
None	3.010	−2.000	0.85		
D.—$MgCl_2$	3 080	"		1.4	2.0
C.—$MgCl_2$	3.113	"		1.2	1.7
D.—NaCl	3.109	"		1.8	2.6
C.—NaCl	3.133	"		1.4	1.9
D.—$MgSO_4$	3.140	"		2.4	3.5
C.—$MgSO_4$	3.196	"		2.1	3.0
D.—K_2SO_4	3.189	"		3.3	4.7
C.—K_2SO_4	3.256	"		2.8	3.9
None	3.074	−1.900	1.06		
D.—$MgCl_2$	3.167	"		1.7	2.2
C.—$MgCl_2$	3.199	"		1.4	1.8
D.—NaCl	3.194	"		2.2	2.9
C.—NaCl	3.216	"		1.6	2.0
D.—$MgSO_4$	3.223	"		2.8	3.6
C.—$MgSO_4$	3.275	"		2.3	2.9
D.—K_2SO_4	3.270	"		3.6	4.7
C.—K_2SO_4	3.329	"		2.9	3.7
None	3.245	−1.700	1.17		
D.—$MgCl_2$	3.328	"		1.5	1.8
C.—$MgCl_2$	3.370	"		1.4	1.6
D.—NaCl	3.362	"		2.1	2.5
C—NaCl	3.390	"		1.6	1.9
D.—$MgSO_4$	3.394	"		2.7	3.2
C.—$MgSO_4$	3.431	"		2.0	2.4
D.—K_2SO_4	3.438	"		3.5	4.2
C.—K_2SO_4	3.497	"		2.8	3.3
None	3.391	−1.500	0.92		
D.—$MgCl_2$	3.488	"		1.7	2.0
C.—$MgCl_2$	3.534	"		1.5	1.7
D.—NaCl	3.522	"		2.3	2.7
C.—NaCl	3.553	"		1.7	2.0
D.—$MgSO_4$	3.542	"		2.6	3.0
C.—$MgSO_4$	3.586	"		2.1	2.4
D.—K_2SO_4	3.588	"		3.5	4.0
C.—K_2SO_4	3.649	"		2.8	3.2

* The asterisk on values of $(b-a)/c$ indicate that those solutions were 0.01 M per 2500 gm. (*i.e.*, 2.50 per cent). All other solutions were 0.005 M per 2500 gm. (*i.e.*, 1.25 per cent).

TABLE IV—*Continued.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	3.573	−1.300	1.12		
D.—$MgCl_2$	3.671	"		1.7	1.8
C.—$MgCl_2$	3.705	"		1.4	1.5
D.—NaCl	3.706	"		2.3	2.5
C.—NaCl	3.742	"		1.8	1.9
D.—$MgSO_4$	3.720	"		2.5	2.7
C.—$MgSO_4$	3.756	"		1.9	2.1
D.—K_2SO_4	3.764	"		3.3	3.6
C.—K_2SO_4	3.813	"		2.5	2.7
None	3.742	−1.100	1.4		
D.—$MgCl_2$	3.842	"		1.6	1.8
C.—$MgCl_2$	3.871	"		1.3	1.4
D.—NaCl	3.876	"		2.2	2.4
C.—NaCl	3.911	"		1.7	1.8
D.—$MgSO_4$	3.882	"		2.3	2.5
C.—$MgSO_4$	3.916	"		1.8	2.0
D.—K_2SO_4	3.926	"		3.0	3.3
C.—K_2SO_4	3.971	"		2.1	2.4
None	3.791	−1.000	1.4		
D.—$MgCl_2$	3.898	"		1.7	1.8
C.—$MgCl_2$	3.930	"		1.4	1.5
D.—NaCl	3.930	"		2.2	2.3
C.—NaCl	3.969	"		1.8	1.9
D.—$MgSO_4$	3.947	"		2.5	2.6
C.—$MgSO_4$	3.976	"		1.9	2.0
D.—K_2SO_4	3.987	"		3.2	3.3
C.—K_2SO_4	4.028	"		2.4	2.5
None	3.973	−0.800	1.5		
D.—$MgCl_2$	4.077	"		1.6	1.6
C.—$MgCl_2$	4.099	"		1.2	1.2
D.—NaCl	4.113	"		2.2	2.3
C.—NaCl	4.138	"		1.6	1.6
D.—$MgSO_4$	4.116	"		2.2	2.3
C.—$MgSO_4$	4.141	"		1.6	1.6
D.—K_2SO_4	4.160	"		4.4	4.5
C.—K_2SO_4	4.190	"		3.1	3.2
None	4.14	−0.600	1.0		
D.—$MgCl_2$	4.256	"		1.6	1.6
C.—$MgCl_2$	4.267	"		1.7	1.7

TABLE IV—*Continued.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S_s
D.—NaCl.	4.295	−0.600		2.2	2.3
C.—NaCl	4.304	"		1.5	1.5
D.—$MgSO_4$..........	4.29	"		2.2	2.4
C.—$MgSO_4$..........	4.30	"		1.5	1.5
D.—K_2SO_4..........	4.328	"		2.6	2.7
C.—K_2SO_4..........	4.349	"		1.9	1.9
None................	4.41	−0.300	1.1		
D.—$MgCl_2$..........	4.512	"		1.3	1.3
C.—$MgCl_2$..........	4.503	"		0.8	0.8
D.—NaCl...........	4.554	"		1.9	1.9
C.—NaCl...........	4.566	"		1.4	1.4
D.—$MgSO_4$	4.556	"		2.0	2.0
C.—$MgSO_4$...	4.542	"		1.1	1.1
D.—K_2SO_4.....	4.596	"		2.5	2.5
C.—K_2SO_4	4.607	"		1.8	1.8
None	4.7	0	1.0		
D.—$MgCl_2$	4.847	"		>1	>1
C.—$MgCl_2$..........	4.827	"		<1	<1
D.—NaCl...........	4.891	"		>2	>2
C.—NaCl...........	4.881	"		<2	<2
D.—$MgSO_4$..........	4 855	"		>2	>2
C.—$MgSO_4$..........	4.859	"		<2	<2
D.—K_2SO_4	4.921	"		>2	>2
C.—K_2SO_4..........	4.898	"		<2	<2
None................	5.1	0.300	0.4		
D.—$MgCl_2$..........	5.28	"		(2.7)	(2.7)
C.—$MgCl_2$..........	5.23	"		(1.7)	(1.7)
D.—NaCl...........	5.38	"		(4.0)	(4.0)
C.—NaCl	5.31	"		(1.7)	(1.7)
D.—$MgSO_4$..........	5.30	"		(2.7)	(2.7)
C.—$MgSO_4$..........	5.26	"		(1.7)	(1.7)
D.—K_2SO_4..........	5 38	"		(4.0)	(4.0)
C.—K_2SO_4..........	5.36	"		(2.5)	(2.5)
None................	6.10	0.500	0.17		
D.—$MgCl_2$..........	6.019	"		−1.2	−1.2
C.—$MgCl_2$	5.960	"		−1.4	−1.4
D.—NaCl	6.055	"		−0.8	−0.8
C.—NaCl	5.977	"		−1.2	−1.2
D.—$MgSO_4$..........	6.033	"		−1.1	−1.1
C.—$MgSO_4$..........	6.004	"		−1.0	−1.0
D.—K_2SO_4	6.07	"		−0.5	−0.5
C.—K_2SO_4..........	6.02	"		−0.8	−0.8

TABLE IV—*Continued.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	6.82	0.600	0.12		
D.—$MgCl_2$	6.61	"		−3.3	−3.3
C.—$MgCl_2$	6.51	"		−3.1	−3.1
D.—NaCl	6.68	"		−2.2	−2.2
C.—NaCl	6.63	"		−1.9	−1.9
D.—$MgSO_4$	6.48	"		−5.4	−5.4
C.—$MgSO_4$	6.65	"		−1.7	−1.7
D.—K_2SO_4	6.65	"		−2.7	−2.7
C.—K_2SO_4	6.45	"		−3.7	−3.7
None	7.72	0.700	0.11		
D.—$MgCl_2$	7.17	"		−9.1	−9.1
C.—$MgCl_2$	7.49	"		−2.3	−2.3
D.—NaCl	7.54	"		−3.0	−3.0
C.—NaCl	7.56	"		−1.6	−1.6
D.—$MgSO_4$	7.53	"		−3.1	−3.1
C.—$MgSO_4$	7.65	"		−0.6	−0.6
D.—K_2SO_4	7.58	"		−2.3	−2.3
C.—K_2SO_4	7.61	"		−1.1	−1.1
None	8.66	0.800	0.12		
D.—NaCl	8.46	"		−3.4	−3.4
C.—NaCl	8.48	"		−1.9	−1.9
D.—K_2SO_4	8.49	"		−2.9	−2.9
C.—K_2SO_4	8.56	"		−1.0	−1.0
None	9.414	0.900	0.19		
D.—NaCl	9.209	"		−3.6	−3.8
C.—NaCl	9.214	"		−2.1	−2.2
D.—K_2SO_4	9.297	"		−2.0	−2.1
C.—K_2SO_4	9.235	"		−1.9	−2.0
None	9.792	1.000	0.26		
D.—NaCl	9.592	"		−3.6	−3.9
C.—NaCl	9.580	"		−2.3	−2.5
D.—K_2SO_4	9.646	"		−2.6	−2.9
C.—K_2SO_4	9.629	"		−1.8	−2.0
None	10.408	1.300	0.57		
D.—NaCl	10.254	"		−2.9	−3.4
C.—NaCl	10.237	"		−2.0	−2.4
D.—K_2SO_4	10.303	"		−2.0	−2.4
C.—K_2SO_4	10.266	"		−1.6	−1.9

TABLE IV—*Concluded.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	10.573	1.400	0.40		
D.—NaCl	10.434	"		−2.6	−4.3
C.—NaCl	10.410	"		−1.9	−3.0
D.—K_2SO_4	10.476	"		−1.8	−3.1
C.—K_2SO_4	10.456	"		−1.3	−2.1
None	11.442	2.000*	0.18		
D.—NaCl	11.390	"		−1.3	(−6)
C.—NaCl	11.307	"		−1.1	(−4)
D.—K_2SO_4	11.410	"		−0.8	−2.6
C.—K_2SO_4	11.384	"		−0.8	−2.8

(0.01 M or 2.5 per cent). At each equivalent of base $(b - a)/c$ which was studied, two solutions were made up without salt, also one "dilute" (0.0375μ) and one "concentrated" (0.0750μ) of each of the salts: NaCl, $MgCl_2$, K_2SO_4, and $MgSO_4$. Each solution contained 5 cc. of a mother solution of gelatin (of twice the concentration) and was made up to 10 cc. All readings at a given value of $\frac{b-a}{c}$ were taken within a few minutes of each other to reduce experimental error in the relative values to a minimum. One solution without salt was measured first and the other last, those with salt coming in between. The same two bubbling water-jacketed hydrogen electrode cells[12] were used in all measurements. Saturated KCl junction was assumed constant. The pH standard was 0.100 M HCl equals pH 1.075 at 25°C. The data are given in Table IV. The two values without salt are averaged in each case where there was any difference.

The values of b' used in equation (7) to calculate values of S' and S were calculated by the formula as described previously.[13]

$$b' = \frac{b-a}{c} + h - \frac{oh}{c}$$

[12] Simms, *J. Am. Chem. Soc.*, 1923, xlv, 2503.
[13] Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

VIII.

SUMMARY.

2.5 and 1.25 per cent gelatin have been titrated potentiometrically in the absence of salts and in the presence of two concentrations (0.0750 and 0.0375μ) of NaCl, $MgCl_2$, K_2SO_4, and $MgSO_4$. The data have been used to calculate values of $\pm S = \nu^{z} - (\nu - 1)^{z}$, where $\nu^{z} = \nu^2 - (\nu^2 - \nu)\, r_x/18$.

The maximum and minimum values of S with NaCl were used to calculate the mean distance (r_x) between like charges in gelatin. This is found to be 18 Å.u. or over (between acid or basic groups) which agrees with the probable value and the titration index dispersion. Thus the data with NaCl are shown to be normal and to obey the equation found to hold for simple weak electrolytes; namely, $pK' - pK = Sa\sqrt{\mu}$ where S is related to the valence and distance by the above equations.

Using the NaCl data as a standard the deviations (ΔS) produced by the other salts are calculated and are found to agree quantitatively with the deviations calculated from equations derived for the simple weak electrolytes. This shows that in gelatin, as in the simple electrolytes, the deviations are related to the "apparent valences" (values which are a function of the true valence and the distance between the groups).

The maximum "apparent valences" of gelatin are 2.4 for acid groups (in alkaline solution) and 1.8 for basic groups (in acid solution). These values correspond to the hypothetical condition of zero distance between the groups. They have no physical significance but have a practical utility first as mentioned above, and second in that they may be used in the unmodified Debye-Hückel equation to give the maximum effect of gelatin on the ionic strength. The true effect is probably even lower than these values would indicate.

The data indicate that gelatin is a weak polyvalent ampholyte having distant groups and that the molecule has an arborescent structure with interstices permeated by molecules of the solvent and other solutes. The size and shape probably vary with the pH.

THE NATURE OF THE IONIZABLE GROUPS IN PROTEINS.

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I.

Dissociation Indices of Amino Acids and Peptides.

In Table I will be seen values of the dissociation indices[1] of various divalent amino acids and peptides (*i.e.*, having one free α-amino group and one free α-carboxyl group).

In Table II are the dissociation indices of some trivalent amino acids and peptides which, in addition to the α-amino group and α-carboxyl group, have a third ionizable group.

The various theories of protein structure now under discussion are all in agreement with the assumption that one amino group and one carboxyl group of each amino acid in a protein molecule are bound in some manner which prevents them from ionizing.[2]

Hence we would expect the ionizable groups[3] *in the protein molecule*

[1] The term "index" is used here, as previously defined, to indicate the negative logarithm of a value; *e.g.*, "dissociation index" = pK = $-\log K$, or "titration index" = pG = $-\log G$.

[2] The "chain" theory, however, assumes that in each protein molecule one α-amino group and one α-carboxyl group are free; but such an assumption is unwarranted by experimental accuracy even if the chain theory were known to be correct and the chains had no branches. In gelatin it would raise the predicted sum of groups from 4.00 to 4.08 which would be in better agreement with the experimental value of 4.05. In egg albumin it would raise the basic groups from 1.6 to 1.75 which would better agree with the experimental value of 2.1. But despite the better agreement it is preferable to simplify our assumptions and attribute the ionizable groups to only the extra groups of trivalent amino acids.

[3] There is still a school of chemists who believe that the charges on a protein molecule are not due to the ionizable groups but to its charge as a colloidal particle. In this article, however, we feel justified in making the unqualified assumption that the charges *in dilute solution* are due to free ionizable groups.

to be due to the "extra" groups in the trivalent amino acids (Table II). It does not follow, however, that the bound groups are always the α groups. For instance in lysine we can conceive of the α-carboxyl and the ϵ-amino group being bound, leaving the α-amino group free to ionize. The titration index will be different than if the α-amino group were bound and the ϵ-amino group were free. However, the data indicate that the latter structure prevails. The same is true of arginine and histidine; the α-amino groups are bound and the extra basic groups are free (in so far as they exist in that form, see below).

TABLE I.

Titration Indices of Divalent Amino Acids and Peptides (and Related Monovalent Electrolytes).

Substance	pG_1' ($-COOH$)	pG_2' ($-NH_2$)	Author*
Acetic acid	$4.740-0.9a\sqrt{\mu}$		S.
Glycollic acid	3.82		S.
Amino ethanol		$9.470+0.5a\sqrt{\mu}$	S.
Glycine ethyl ester hydrochloride		7.655	S.
Glycine	$2.365-0.08a\sqrt{\mu}$	$9.715-a\sqrt{\mu}$	S.
Alanine	2.35	9.72	L.S.
Sarconine	2.23	10.01	L.S.
Tryptophane	2.266	9.372	S.
Valine	2.28	9.65	L.B.
Glycyl-glycine	3.12	8.07	L.S.
Sarcosyl-glycine	3.10	8.51	L.S.
Glycyl-sarconine	2.83	8.54	L.S.
Sarcosyl-sarconine	2.86	9.10	L.S.
Alanyl-alanine	3.17	8.42	L.S.
Glycyl-valine	2.28	8.30	L.B.
Glycyl-leucine	3.18	8.29	S.
Glycyl-alanine	3.15	8.25	S.
Glycyl-asparagine	2.9	8.3	S.
Glycyl-glycyl-glycine	3.26	7.91	L.S.
Glycyl-alanyl-alanyl-glycine	3.30	7.9	L.S.
Alanyl-alanine anhydride (enol group = 13.5)			L.B.

* L.S. represents Levene and Simms; L.B., Levene and Bass; and S., Simms.

TABLE II.

Titration Indices of Trivalent Amino Acids.

Substance	pG′ α−COOH	pG′ α−NH₂	Third group: Group	Third group: pG′	Approximate pG′ in protein	Author*
Aspartic acid	$2.05-0.6a\sqrt{\mu}$	$10.00-2.3a\sqrt{\mu}$	−COOH	$3.87-1.2a\sqrt{\mu}$	3.5	S.
Glycyl aspartic acid	2.81	8.60	−COOH	4.45		S.
Aspartyl glycine	2.10	9.07	−COOH	4.53		L.S.
Glutamic acid	2.11	9.45	−COOH	4.06		L.S.
Histidine	1.46	9.41	=NH	6.06	6.1	S.
Arginine	2.29	9.64	=NH	8.15	8.1	S.
Tyrosine	2.24	10.28	−OH	9.21	9.4	S.
Lysine	2.04	9.06	$-NH_2$	10.45	10.6	L.S

* L.S. represents Levene and Simms; and S., Simms.

TABLE III.

Content of Amino Acids in Gelatin.

Amino acid		Weight	Molecular weight	Mols per 61,500 gm.	Mols per 2500 gm.
		per cent			
Divalent	Glycine	25.5	75	209	8.5
	Alanine	8.7	89	60	2.4
	Leucine	7.1	131	33	3.4
	Serine	0.4	105	2	0.1
	Phenylalanine	1.4	165	5	0.2
	Proline	9.5	115	51	2.1
	Oxyproline	14.1	131	66	2.7
Total divalent................		66.7		426	17.4
Trivalent	Aspartic acid	3.4	133	16	0.64
	Glutamic acid	5.8	147	24	1.0
	Histidine	0.9	155	4	0.15
	Arginine	8.2	174	29	1.2
	Tyrosine	0.01	181	0.03	0
	Lysine	5.9	146	25	1.0
Total trivalent.........		24 2		98	4.0
Total amino acids........... .		90.9		525	21.4

II.

The Predicted Ionizable Groups of Proteins.

In Table III are the percentages of various amino acids in gelatin as found by Dakin.[4] Since we are interested only in the trivalent amino acids these are listed in Table IV in the column of "predicted" equivalents (per 2500 gm., an arbitrary unit weight). The third column gives the predicted values for gelatin and the eighth column for egg albumin.

TABLE IV.

Equivalents (per 2500 gm.) of the groups found, compared with the equivalents predicted from the content of the respective amino acids. The values for gelatin must be multiplied by 24.6 to obtain the equivalents per molecular weight of 61,500 (Kunitz); and the egg albumin values should be multiplied by 13.5 for molecular weight of 33,800.

1		2	3	4	5	6	7	8	9	10
			Gelatin					Egg albumin		
				1st titration		2nd titration				
Groups	Sources	Approximate indices*	Predicted	Found	Difference	Found	Difference	Predicted	Found	Difference
Acidic	Dicarboxylic acids	$pG_1' = 3.5$*	1.65	1.75	+0.1	1.75	+0.1	?	1.6	?
	Tyrosine	$pG_5' = 9.4$	0	0	0	0	0	0.6	0.4	−0.2
Basic	Unknown	$pG_2' = 4.6$	0	1.0	+1.0	1.05	+1.05	0	0.9	+0.9
	Histidine	$pG_3' = 6.1$	0.15	0.2	0.05	0.15	0	0.3	0.3	0
	Arginine	$pG_4' = 8.1$	1.2	0.2	−1.0	0.2	−1.0	0.7	0.3	−0.4
	Lysine	$pG_6' = 10.6$*	1.0	0.9	−0.1	0.9	−0.1	0.6	0.6	0
Total acid groups ("base-binding capacity")			1.65	1.75	+0.1	1.75	+0.1	?	2.0	?
Total basic groups ("acid-binding capacity")			2.35	2.30	−0.05	2.30	−0.05	1.6	2.1	+0.5
Sum of arginine + "4.6 group"			1.2	1.2	0	1.25	0.05	0.7	1.2	0.5

* The titration indices (pG′ values) found, were those given above except in three cases: pG_1' in egg albumin is 2.9 (instead of 3.5). pG_6' in egg albumin was 10.8, and in the first titration of gelatin was 10.4 (instead of 10.6 as found in the second titration).

[4] Dakin, *J. Biol Chem.*, 1920, xliv, 499.

In Fig. 1, Curve *B*, we see the way the titration curve of gelatin would look if these groups occurred in gelatin in the quantities "predicted" by the amino acid content. The isoelectric point would be around pH 8.0 instead of 4.7. The experimental curve (*A*) of gelatin has an isoelectric point at 4.7 and has a different shape between pH 4 and 10.

We have analyzed the experimental curve of gelatin (Curve *A*, Fig. 1) in order to see if it could be resolved into groups with the

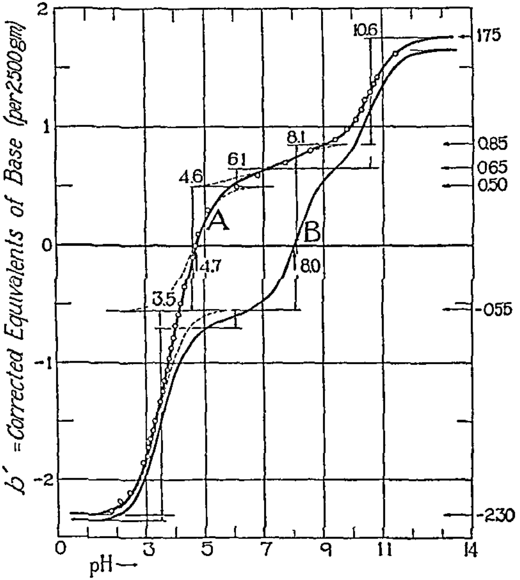

FIG. 1. Gelatin titration curves. Curve *A*. Experimental points and curve corresponding to values marked "found" in Table IV. Curve *B*. Curve "predicted" from trivalent amino acids (see Table IV).

indices given in Table IV. Our method was similar to that of E. J. Cohn[5] except that we restricted ourselves to titration indices which the free ionizable groups should have in the protein molecule, and furthermore we did not consider the isoelectric point as a transition between one ionizable group and another. It has been shown by Northrop[6] that gelatin contains at least two constituents not chemically bound. It is probable that gelatin and other proteins consist of mixtures of numerous constituents. In speaking of the "gelatin molecule" we recognize that we are dealing with a mixture of different molecules. This is kept in mind in the construction of the "predicted" curve and the analysis of the experimental curve. The isoelectric points do not represent transition points between ionic species as is the case with pure chemical substances having groups close together.

III.

The Actual Groups of Proteins.

The analysis of the experimental gelatin curve (Fig. 1, Curve *A*, and Columns 4 and 6 of Table IV) shows that the total number of acid groups agrees within 0.1 equivalent (per 2500 gm.) with the value predicted from the content of acidic amino acids.

The total number of basic groups agrees (within 0.05 equivalent) with the predicted value.[7] However, the quantity of arginine is a whole equivalent (1.0 eq.) too low in quantity. Furthermore, *the deficiency in arginine is compensated by the existence in gelatin of one equivalent of a basic group of unknown source with a titration index* (pG'_3) *at 4.6.*

A glance at the last three columns of Table IV shows that the same is true of egg albumin (see Fig. 2). Arginine group is 0.4 equivalent

[5] Cohn, *Physiol Rev.*, 1925, v, 349.

[6] Northrop, *J. Gen. Physiol.*, 1926–27, x, 161.

[7] This agreement is not found if we use the content of histidine found by Van Slyke (0.5) instead of the value of Dakin (0.15). The latter value agrees with the amount of histidine group (0.2) in gelatin. However, the relation between arginine and 4.6 group is not affected by the value assumed for histidine.

too low, while there is 0.9 equivalent of the "4.6 group" in egg albumin.[8]

The nature of the "4.6 group" is unknown. It is undoubtedly a basic (amino) group. The aliphatic amino groups have indices which range from 12.0 for diethyl amine to 7.65 for glycine ester hydrochloride. Unsaturated bases are weaker: guanidine has a basic group at 6.0; arginine at 8.15, and histidine at 6.06. However, bases as weak as 4.6 are not found except in the very unsaturated systems of aniline (4.6), cytosine (4.6), and isocystosine (4.0). The latter bases have an amino group attached to a conjugated unsaturated cyclic system.

Hence we are safe in concluding that a basic group with a conjugated unsaturated (and perhaps cyclic) structure occurs in gelatin

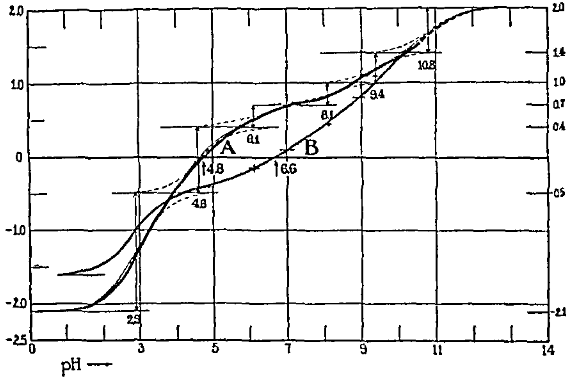

Fig. 2. Egg albumin titration curves. Curve *A*. Experimental curve (heavy line) and "found" curve (light line, see Table IV). Curve *B*. Curve "predicted" from trivalent amino acids.

[8] It would be interesting to know if the discrepancy of 0.5 eq. in the sum of arginine plus 4.6 group in egg albumin is due to an error in the arginine value found on hydrolysis (Column 8). In a subsequent paper it will be shown that the 2.2 eq. of arginine derived from edestin exist as "prearginine."

and egg albumin and that this group is disrupted on hydrolysis. It seems likely that this 4.6 group produces arginine on hydrolysis.

It will be noted that the agreement between the deficiency of arginine and the amount of the 4.6 group is quantitative in the case of gelatin while there is a discrepancy of +0.5 equivalent for egg albumin. Hence we must consider the alternative hypothesis, namely, that the 4.6 group does not produce arginine on hydrolysis and that part of the arginine group in the protein molecule is bound in some unknown manner so that it does not ionize. This seems unlikely, however and does not agree with the data on edestin.[8]

Since the material in proteins which dissociates as a weak base at pH 4.6 appears to give arginine on hydrolysis, we will refer to it as "*prearginine.*"

IV.

Deaminized Gelatin.

Hitchcock[9] showed that the loss of nitrogen on deamination of gelatin agreed with the decrease in acid-combining capacity. It seemed desirable to determine which amino groups in gelatin are removed on deamination. We therefore prepared some deaminized gelatin and titrated it.

If we compare the curve of deaminized gelatin in Fig. 3*A* with that of gelatin it is obvious that the process of deamination has principally removed the free lysine group and that the prearginine, the arginine, and the histidine groups are not materially affected.

In order to analyze the data more accurately we drew the comparison curve,[10] C_1 in Fig. 3*B*. The drop at 10.6 indicates that there is less of the lysine group in the deaminized gelatin. Adding 0.75 equivalent of 10.6 group (and lowering the curve 0.75 equivalent) gives the second comparison curve, C_2.

This second curve (C_2) represents the relation between deaminized gelatin and gelatin deprived of 0.75 equivalent of its lysine group.

[9] Hitchcock, *J. Gen. Physiol.*, 1923–24, vi, 95.

[10] Simms and Levene, *J. Biol. Chem.*, 1926, lxx, 319; Levene and Simms, *J. Biol. Chem.*, 1926, lxx, 327. This method could have been used in analyzing the data in Fig. 1, but was not adopted since the exact pG′ values were uncertain.

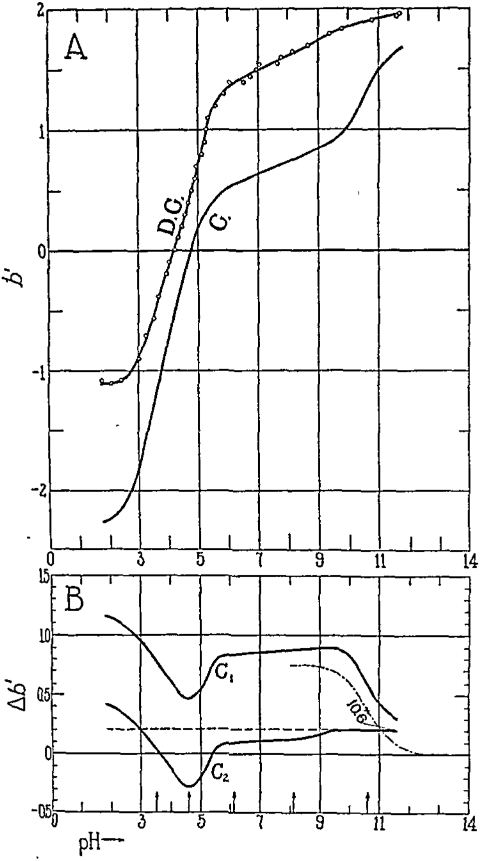

FIG. 3. *A*. Titration curves of gelatin (*G*.) and deaminized gelatin (*D.G.*). *B*. First comparison curve, C_1 (equals *D.G.* minus *G*.); and second comparison curve, C_2 (equals C_1 minus 0.75, plus 0.75 equivalent of 10.6 group).

The drop at 4.6 shows that the index of this group is materially raised (to, say, 5.0 or 5.1) on deamination. There is also a rise of the 6.1 and 8.1 indices and a drop in the acid carboxyl group index (3.5). The height of the curve at pH 11 shows that there is 0.2 equivalent more carboxyl group than before deamination (perhaps due to slight hydrolysis followed by deamination of the amino group produced).

The important points to observe are that *deamination removes a large part of the lysine group* and *does not remove the prearginine, the arginine, or the histidine groups.*

This agrees with the observation of Van Slyke and Birchard[11] that the amino nitrogen of proteins equals half the lysine nitrogen.

TABLE V.

Titration of Deaminized Gelatin, 0.0100 M per 2500 Gm. (2.5 per cent).

pH	$\frac{b-a}{c}$	b'	pH	$\frac{b-a}{c}$	b'
A. More acid solutions.					
1.773	−3.000	−1.083	4.347	.100	.105
2.103	−2.000	−1.105	4.471	.200	.204
2.431	−1.500	−1.081	4.565	.300	.303
3.005	−1.000	−0.889	4.660	.400	.402
3.223	−0.800	− .733	4.802	.500	.502
3.521	− .600	− .567	4.888	.600	.601
3.670	− .400	− .377	4.936	.700	.701
3.962	− .200	− .188	5.101	.800	.801
4.033	− .100	− .090	5.228	.900	.901
4.213	0	+0.006	5.280	1.000	1.001
4.217	0	.006	5.331	1.100	1.101
4.341	0.100	.105			
B. More alkali solutions.					
5.585	1.200	1.200	7.769	1.600	1.600
5.856	1.300	1.300	8.145	1.650	1.650
(6.52)	1.400	1.400	8.648	1.700	1.694
(6.07)	1.400	1.400	9.373	1.800	1.796
6.746	1.450	1.450	9.785	1.850	1.841
6.964	1.500	1.500	10.771	2.000	1.913
(7.07)	1.550	1.550	11.570	2.500	1.944
(7.67)	1.550	1.550	11.693	2.700	1.962

[11] Van Slyke and Birchard, *J. Biol. Chem.*, 1913, xvi, 539.

V.

Hydrolyzed Deaminized Gelatin.

An attempt was made to partially hydrolyze some deaminized gelatin with HCl and determine if the 4.6 group remained intact. The readings were very unsatisfactory and it was not possible to draw any conclusion.

VI.

EXPERIMENTAL.

The data for gelatin are the data without salt in the preceding article of this series.[12] They do not differ materially from the corrected data obtained by others on direct titration of gelatin.

The deaminized gelatin was obtained by treating 100 gm. of isoelectric gelatin in 1 liter of water with 10 gm. of solid $NaNO_3$ and about 15 cc. of glacial acetic acid, heating on steam bath (with occasional stirring) for 4 hours, and then dialyzing through collodion membranes by a method described in another article.[13] The material after 24 hours had a conductivity of 6.4×10^{-5} reciprocal ohms (for 6.7 per cent protein).

This material was titrated by methods previously described.[14] The data are given in Table V.

The experimental curve for egg albumin is taken from the compiled data of Cohn.[5]

VII.

SUMMARY.

Analysis of the experimental titration curves shows that gelatin contains acid groups with dissociation indices at pH 2.9 to 3.5 corresponding quantitatively with the content in dicarboxylic amino acids; and that the acidic group at pH 9.4 in egg albumin agrees with the amount of tyrosine.

[12] Simms, *J. Gen. Physiol.*, 1927–28, xi, 613.
[13] Kunitz and Simms, *J. Gen. Physiol.*, 1927–28, xi, 641.
[14] Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

The amounts of histidine and lysine present in both these proteins agree quantitatively with basic groups at pH 6.1 and pH 10.4 to 10.6, respectively.

However, the quantity of the arginine group (pH 8.1) in these proteins is considerably less than the amount of arginine found on hydrolysis. This deficiency is compensated (quantitatively with gelatin and approximately with egg albumin) by a basic group at pH 4.6.

The structure of this "4.6 group" should be similar to aniline and cytosine in consisting of an amino group on a conjugated unsaturated (perhaps cyclic) system. It would appear that the 4.6 group is disrupted on hydrolysis, producing arginine, and may be referred to as "prearginine."

The presence of prearginine in proteins, instead of the full amount of arginine, has an important effect on the properties. Otherwise the isoelectric point of gelatin would be 8.0 (instead of 4.7) and of egg albumin 6.6 (instead of 4.8), and the titration curves would be quite different in shape between pH 4 and 10.

Deamination of gelatin produces no decrease in prearginine, arginine, or histidine groups, but removes nearly all of the lysine group.

DIALYSIS WITH STIRRING.

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(Accepted for publication, December 22, 1927.)

The separation of the diffusible from the non-diffusible substances by dialysis through a collodion membrane is a slow process when the solution remains stagnant even though fresh water be circulated on the other side of the membrane. This has led to the adoption of electrodialysis, which has proved successful in some cases, but which has the disadvantage of complexity and of electrolysis.

In order to hasten diffusion, Northrop and Kunitz[1] adopted the method of placing toy glass "marbles" in their collodion bags. The bags were immersed in water in glass tubes which were rocked mechanically, causing the marbles to roll from end to end of the bags. This method of stirring has been slightly modified for the purification of proteins. The apparatus is shown in Fig. 1. Collodion bags are made as described by Northrop and Kunitz.[1] These are placed in the tubes and distilled water of any desired temperature is allowed to circulate past them while they are rocked by means of an automobile windshield scraper.

The effect of stirring was determined by filling two membranes with 0.5 M NaCl and measuring the rate of diffusion with a flow of distilled water of 8 cc. per tube per minute. One membrane was rocked, the other was stationary. The results are given in Table I.

A thick suspension of egg albumin crystals in concentrated ammonium sulfate was dialyzed in a similar apparatus placed in a cold bath (5°C.) with a flow of 10 cc. distilled water per tube per minute. The results are given in Table II.

The completeness of the dialysis is shown by the specific conductivity of 8.2×10^{-6} reciprocal ohms after 48 hours. (The protein concen-

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 351.

tration was 14 per cent by the dry weight method.) A second batch gave 2.4×10^{-5} after 96 hours dialysis with shaking (17.1 per cent protein). This compares favorably with the value of 2.1×10^{-5} obtained by Pauli[2] (on 4 per cent albumin) after long dialysis followed by electrodialysis. The value obtained by Svedberg and Nichols[3] on egg albumin which was dialyzed in flowing distilled water for 18 days and subsequently electrodialyzed for about 2 days, was 3.42×10^{-5} for a 6.6 per cent solution.

TABLE I.

Effect of Stirring on Diffusion of 0.5 M NaCl.

Time of dialysis	Specific conductivity × 1,800	
	Stirred	Not stirred
hrs.		
0	100	100
2	5	27
4	0.3	9
6	0.1	1.4

TABLE II.

Dialysis of Egg Albumin in Concentrated Ammonium Sulfate, While Stirring.

Time of dialysis	Specific conductivity × 10^{-5}
hrs.	
4	820
7	210
24	18
31	16
48	8

Deaminized gelatin containing a large amount of electrolyte was dialyzed to 6.4×10^{-5} reciprocal ohms (6.7 per cent protein) in 24 hours in this apparatus.

Description of Apparatus.

The apparatus is shown in Fig. 1 and does not require detailed description. Distilled water is supplied by a bath which may be kept

[2] Pauli, W., *Biochem. Z.*, 1925, clxiv, 400.

[3] Svedberg, T., and Nichols, J. B., *J. Am. Chem. Soc.*, 1926, xlviii, 3081.

at higher or lower or at constant temperature as desired. The water siphons down the inlet tube into the three glass tubes each containing four stoppered collodion sacs filled with the solution and containing a glass marble. Each sac holds 30 to 35 cc. of solution. The water then flows out the outlet tube. The flow of water is regulated with a screw clamp.

In the case of solutions not affected by air, a large bubble of air may be left in the bags in place of the marbles.

The glass tubes are attached with copper wire to a board which hinges on two screws and is rocked by means of a windshield scraper.

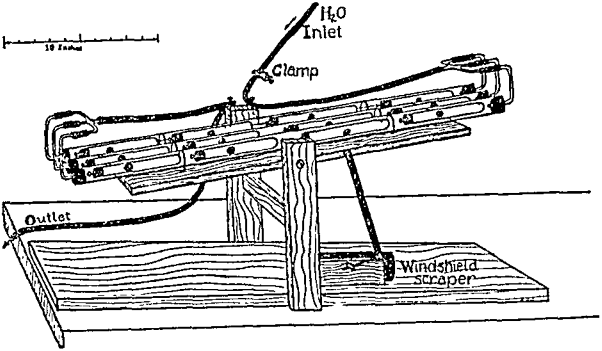

FIG. 1.

The rate of rocking is adjusted to a speed such that the marbles pass from one end of the sacs to the other (*i.e.*, 1 cycle in about 6 seconds).

The rate of flow of water is about 8 cc. per tube per minute. If the concentration of dialysate is high 2 to 3 times as much water should be used for the first 2 to 3 hours. For running overnight a large amount of water is required. If a wash-boiler or other tank is not enough, two or more 5 gallon bottles (on the same level) may be connected to it with large siphon tubes.

The windshield scraper is better adapted to most laboratory uses if the axis of the arm is turned 90° from its original position. This may be done by removing the shaft and filing or drilling it to make a seat for the set screw 90° from the original position.

SUMMARY.

Substances to be purified by dialysis are placed in collodion bags together with a toy "marble" or a bubble of air. The bags are stoppered and placed in glass tubes of a rocking machine. Distilled water of the desired temperature is circulated through the tubes (around the bags) at a rate of about 8 cc. per minute per bag while the machine is in motion. The rolling of the marbles or bubbles causes stirring which makes it possible to remove the salts from a protein solution in 24 to 48 hours.

THE METABOLISM OF LIVER TISSUE FROM RATS OF DIFFERENT AGES.

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Accepted for publication, February 18, 1928.)

Falk, Noyes and Sugiura (1) have studied the lipase or ester-hydrolyzing actions of aqueous extracts of the whole rat at different ages covering the life cycle from 3 days before birth until the age of 3 years, and presented the results in the form of curves. They found that the curves for the embryo and youngest rats approached those given by the Flexner-Jobling carcinoma, changing with the age of the rat to the type characteristic of the adult rat and appearing to revert again to some extent to the embryonic type for the oldest rats. Similar experiments with the protease actions of the extracts of whole rats of different ages on three protein preparations did not give differences similar to those found for the lipase actions.

Warburg (2) and his associates as a result of an extensive study of metabolism of tissues consider that they can detect four distinct types, namely; normal resting tissue with a slight anaerobic glycolysis and a high respiratory rate; embryonic tissue with a high respiratory rate and a high anaerobic but a low aerobic glycolysis; malignant tumor tissue with a low respiration and a high aerobic and anaerobic glycolysis; benign tumor tissue with the same type of metabolism as malignant tissue but with less active glycolytic function. Murphy and Hawkins (3) extended Warburg's experiments and concluded that a classification of tissues on the basis of the type of metabolism does not correspond to the biological groupings but that a classification (4) could be made by measuring the anaerobic glycolytic function of the tissues.

The present experiments were undertaken to determine whether there is any difference in the metabolism of the livers of rats of different ages. The livers of rats in 3 groups were used; Group 1, rats 22

months old; Group 2, adult rats approximately 1 year old; Group 3, rats varying in age from 3 to 21 days old.

The method used was that devised by Warburg, the details of which are described by him in his publications (2) and also by Murphy and Hawkins (3). The results of the experiments are given in Table I.

The results in Table I show that there is no difference in respiration and aerobic glycolysis in the livers of rats of different ages. There is practically no anaerobic glycolytic activity in the livers of the old and normal adult rats, but there is a certain amount in the livers of the

TABLE I.

Average for group	No. of animals	Q_{O_2}	$Q^{O_2}_{CO_2}$	$Q^{N}_{CO_2}$
1	15	−11.5	1.0	0.8
2	12	−9.4	0.4	2.0
3	15	−13.2	0.0	5.7

Q_{O_2} = Respiration. C.mm. of oxygen consumed per hour by 1 mg. per dried weight of tissue.

$Q^{O_2}_{CO_2}$ = Aerobic glycolysis. C.mm. of carbon dioxide produced by glycolysis per hour by 1 mg. per dried weight of tissue.

$Q^{N}_{CO_2}$ = Anaerobic glycolysis. C.mm. of carbon dioxide produced per hour by 1 mg. per dried weight of tissue when respiration has been checked by potassium cyanide.

very young rats. This would mean according to Warburg's grouping that the livers of young rats have the same type of metabolism as embryonic tissue. These results would seem to substantiate the conclusions of Hawkins that the glycolytic activity of a tissue is a function of the growth rate, as the growth rate of the livers of the young rats would be greater than that of normal adult or old rats.

Accepting the conclusion that the glycolytic activity of a tissue is a function of its growth rate, these results are in agreement with those of Falk and his associates who found that their curves for embryo and young rats approached those for the Flexner-Jobling tumor.

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THE INFLUENCE OF TEMPERATURE ON THE PHOTOSENSORY LATENT PERIOD.

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(Accepted for publication, December 14, 1927.)

I.

Numerous researches on the photosensory properties of the clam, *Mya arenaria*, and the ascidian, *Ciona intestinalis*, have shown that the photoreceptor system is composed of at least two processes (Hecht, 1925). One way in which this is made evident is by the composition of the reaction time of these animals to light. A short exposure to light is sufficient to elicit the siphon retraction which is the typical response of these animals to illumination. However the response does not take place immediately, but only after a comparatively long latent period during which the animal may remain in the complete absence of light. With an illumination of 1000 meter candles an exposure of 0.01 second is enough to cause a siphon retraction in *Mya* after about 2.0 seconds of latent period. This is a ratio of 1:200 for the durations of exposure and latent periods respectively.

During the exposure period the light produces a photochemical change in a sensitive substance. Experiments to test the interrelations between intensity, time, wave-length, and various conditions of exposure and adaptation of the animals (Hecht, 1925, Piéron, 1926) have shown that this photochemical process is probably a reversible photochemical reaction of a comparatively simple type. Nevertheless the products of this photolytic activity do not directly release the impulse to the associated nerve endings which starts the very rapid train of nervous and muscular events culminating in the siphon

* The experiments here recorded were made in 1924–25 when, as Fellow of the International Education Board, I occupied the Jacques Loeb Memorial Table at the Zoological Station in Naples.

retraction. Between the photochemical effect and the siphon response there is intercalated an additional process which is represented by the latent period.

The long duration of the latent period in comparison to that of the exposure period would by itself indicate the different nature of the two processes. The same thing is evident from the different effects of temperature on exposure period and on the latent period. The former is hardly changed by an increase in temperature, thus corroborating its photochemical nature. The latter, however, in the case of *Ciona* and *Mya* at least, varies with temperature in a way similar to that of chemical reactions uninfluenced by light.

The two processes are intimately related. Conditions like time and intensity which control the photochemical reaction, exert a subsequent influence on the latent period of such a kind that its speed varies with the magnitude of the photochemical change. This has been interpreted as showing that the two processes form a coupled reaction of which the secondary, latent period reaction proceeds only in the presence of products from the primary, photochemical reaction. The mechanism which couples the two reactions may be a catalysis or a direct chemical relation.

The existence of such a coupled series of reactions is fairly common in the light reception of organisms, to judge only by the existence in the reaction time of a primary "light" period and a secondary "dark" period. In the heliotropic curving of plants such a division is of course well known, since the two parts are often widely separated in time, the turning (response) taking place a long time after the exposure. The primary reaction here is photochemical as Blaauw (1908) and Fröschel (1909) showed years ago. In the phototropic turning of sessile animals like *Eudendrium* there is a similar division of "light" and "dark" reactions (Loeb and Wasteneys, 1917). A secondary "dark" period in the phototropism of free moving animals is not often demonstrable, since the orientation of the animal ceases with the removal of the light (Minnich, 1919). Even here, however, Mast (1912) has shown that the fire-fly orients *after* the stimulating light has been extinguished. The latent period is thus an integral part of the process of light reception, and any information that will indicate its nature is relevant to an understanding of the photoreceptor process.

The effect of temperature on the duration of the latent period furnishes information of this kind. In *Mya* the relation between temperature and latent period may be described by the Arrhenius equation; the same is true of *Ciona*. The value of μ in the equation is different in the two cases. Since *Mya* and *Ciona* are the only animals in which the latent period has been studied in this way, I took the occasion of a stay in Naples to study the effect of temperature on the latent period of *Pholas dactylus*, an animal whose photosensory properties greatly resemble those of *Mya* and *Ciona* (Hecht, 1926–27).

II.

The procedure in these experiments is comparatively simple. An animal, in its rectangular dish, is placed in a large water bath where with the help of stirring, it quickly attains the temperature of the bath. Here it is left for about an hour in the dark, during which time it maintains its temperature constant by means of the gentle stirring created by its water current. Its reaction time is then measured after an exposure of 0.05 hm. (= hundredths of a minute) to an illumination of 2000 m.c. This involves taking the animal, in its dish, out of the thermostat and transferring it to the measuring table. The temperature to 0.1°C. is measured immediately before the exposure; but no difference is noted between the temperature then and in the thermostat, because of the volume of water in the dish in which the animal rests. After the exposure the animal is replaced in the water bath, the temperature of which is now rapidly changed. After a few minutes the sea water in the dish has reached the temperature of the bath, and the animal is at that temperature for an hour. Its reaction time is then again measured for the same exposure to the same light. After five such measurements, for a series of decreasing temperatures, five similar measurements are made at intermediate points with a series of increasing temperatures. Only one reading is made with each animal at each temperature.

All the manipulations are carried out in a dark room with the help of a very dim ruby lamp. By the light of such a lamp it is difficult to observe the siphon retraction which occurs some time after the exposure. The procedure is therefore adopted of watching the siphon

as silhouetted against the red light. In this way the very beginning of the retraction may be accurately timed with a stop-watch.

The temperature range over which the measurements are made is limited to about 10°. Below 10°C. the beginning of the siphon retraction ceases to be sharp enough for accurate measurement, while above 20°C. the reaction time becomes too short for timing with a stop-watch.

III.

The experiments were made with eight animals, all of which had been in the dark for about a week before the experiments were begun.

TABLE I.

Relation between the Temperature and the Photosensory Latent Period. Exposure Period is 0.05 Hm. (= Hundredths of a Minute) to an Illumination of 2,000 Meter Candles.

Temperature	Reaction time	Latent period, p	
		Measured	$ln\ p = \frac{18,300}{RT} - 30.95$
°C.	hm.	hm.	hm.
10.5	3.93	3.88	3.75
11.3	3.51	3.46	3.43
12.7	2.94	2.89	2.94
13.3	2.84	2.79	2.75
14.6	2.43	2.38	2.37
15.3	2.24	2.19	2.27
16.2	2.05	2.00	2.00
17.3	1.84	1.79	1.77
18.3	1.64	1.59	1.59
18.4	1.63	1.58	1.57

The data secured are given in Table I, where each value of the reaction time is the average of eight measurements, one with each animal. Table I shows that the effect of temperature on the latent period is of the magnitude of that for chemical reactions. In this respect it resembles what has already been found for the latent period of *Ciona* and *Mya;* and thus supports the general idea of a coupled reaction underlying the photosensory process in these three animals.

To treat the data more quantitatively, I have supposed that the

latent period represents the duration of a chemical reaction which must produce a definite amount of its products in order to set off the stimulus to the sensory nerve. Therefore the reciprocal of the latent period is proportional to the velocity constant of the reaction, and may be used to determine whether the reaction follows the Arrhenius equation

$$ln\ k = -\frac{\mu}{R\ T} + C \tag{1}$$

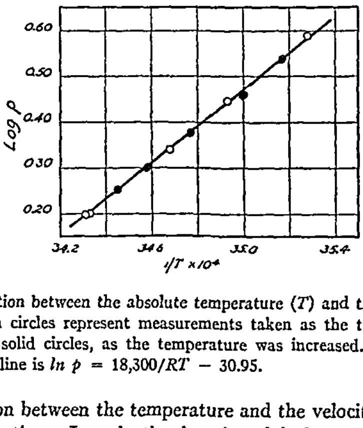

Fig. 1. Relation between the absolute temperature (T) and the latent period (p). The open circles represent measurements taken as the temperature was decreased; the solid circles, as the temperature was increased. The equation of the straight line is $ln\ p = 18{,}300/RT - 30.95$.

for the relation between the temperature and the velocity constant of a chemical reaction. Let p be the duration of the latent period. Then $1/p$ may be substituted for k, and the equation becomes

$$ln\ p = \frac{\mu}{R\ T} - C \tag{2}$$

which, when $ln\ p$ is plotted against $1/T$, gives a straight line whose slope is μ/R. Fig. 1 shows the data of Table I plotted in this manner. It is clear that the Arrhenius equation describes the data with considerable fidelity. Here $\mu = 18{,}300$; R is the gas constant; and C, which equals 30.95, is merely an integration constant. To show the agreement between experimental and calculated values I have in-

troduced in Table I the values of the latent period computed in terms of the equation.

IV.

Two things follow from these temperature data on *Pholas*, *Ciona*, and *Mya*. The first is that the relation between temperature and latent period is expressible in the form of an equation applicable to many chemical reactions and to many biological processes. In terms of the previously suggested hypothesis, it may thus be supposed that the latent period represents the duration of a reaction $L \rightarrow T$ in which an inert substance L is converted into an active substance T, of which a definite amount is required to set off the stimulus to the associated nerve fiber. It is obvious that in such a system the Arrhenius equation should apply with the same accuracy as in an isolated chemical reaction.

TABLE II.

Values of μ for the Effect of Temperature on the Photosensory Latent Period of Different Animals.

Species	μ
Ciona intestinalis	16,200
Mya arenaria	19,700
Pholas dactylus	18,300

The second thing which the data show is that the value of the constant μ is different for each species. Table II gives the relevant information taken from the present data and from previous work (Hecht, 1918–19, 1925–28). Precisely what the specific values of μ mean is uncertain at present. The value $\mu = 19{,}700$ for *Mya* has been found in other instances (Crozier, 1925–26) but is apparently not associated with any definite group of biological or chemical processes. The very common value $\mu = 16{,}200$, found for *Ciona*, occurs in many cases where oxidations seem to be concerned, and is possibly related to the function of iron as catalyst (Crozier, 1924–25).

The value $\mu = 18{,}300$, found here for *Pholas*, is not uncommon in biological processes (Crozier, 1925–26). It is therefore not without interest to note its occurrence in purely chemical reactions in which

iodine is concerned. Plotnikow (1907) found $\mu = 18{,}400$ for the oxidation of HI in the dark. More recently Conant and Hussey (1925) studied the relations between the structure of a large assortment of organic halides and the speeds of their reaction with inorganic iodides like KI and NaI. They found that although the velocity of this metathesis varies enormously with the structure of the organic halide, the effect of temperature on the velocity can be uniformly expressed by the Arrhenius equation when $\mu = 18{,}400$. Similarly, Rideal and Williams (1925) found that the liberation of iodine from KI by ferric salts has a temperature coefficient of $\mu = 18{,}300$.

These similarities between biological and chemical behavior need not be taken as indicating identities. Nevertheless, the data are striking, and any theory which is to account for the effect of temperature on biological activities must be prepared to take them into account. For our present purposes they are significant in suggesting that the differences in μ value for the latent periods of the three species indicate a specific difference; and that the latent period reaction which has been formally expressed as $L \rightarrow T$ is most likely different in each species. In other words, though the organization of the photoreceptor process is the same for all, the materials which make up the reactions are different in the three animals.

SUMMARY.

1. The effect of temperature on the photosensory latent period in *Pholas dactylus* is accurately described by the Arrhenius equation when $\mu = 18{,}300$.

2. The adequacy of this equation has already been found for two other photosensitive animals, *Mya* and *Ciona*, which are very similar in behavior to *Pholas*. The value of μ is different for each of the three species studied.

3. This is taken to mean that though the organization of the receptor process is the same for the three species, the chemical materials concerned are very likely different.

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THE RELATION OF TIME, INTENSITY AND WAVE-LENGTH IN THE PHOTOSENSORY SYSTEM OF PHOLAS.

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I.

A series of studies (Hecht, 1925, 1926–27) of the sensibility to light of a number of animals has brought out the fact that the essentials of the photosensory process are the same in all of them. For example, the reaction time to light of such diverse animals as *Ciona* and *Pholas* and the frog tadpole, is composed of two distinct parts, an exposure period during which the animal must be subjected to the light, and a latent period during which it may be in the dark. Again, the dark adaptation of very different animals presents the same kind of course, and may be regarded as kinetically identical. All such similarities suggest that the organization of the processes which make up the photosensory system is the same in a diversity of animals.

Nevertheless there is evidence to show that although the organization is the same, the materials which compose the reactions, and probably the reactions themselves, are specific. The most quantitative expression of such differences has been found in the effect of temperature on the duration of the latent period. In the three animals in which this has been carefully studied (Hecht, 1927–28) it has been shown that though the relation between temperature and latent period may be expressed accurately by the Arrhenius equation

$$\ln p = \frac{\mu}{R\,T} - C$$

* The experiments here recorded were made in 1924–25 when, as Fellow of the International Education Board, I occupied the Jacques Loeb Memorial Table at the Zoological Station in Naples.

the value of the temperature characteristic μ is different for each species. Here p is the latent period, R is the gas constant, T the absolute temperature, and C an integration constant of no significance. For *Ciona*, μ = 16,200; for *Mya*, μ = 19,700; and for *Pholas*, μ = 18,300, values for some of which counterparts may be found in certain well known chemical reactions and which may probably represent different chemical materials active in the latent period.

The same kind of difference may be shown to exist in the substances concerned with the part of the photosensory process which occurs in the exposure period. It can be shown that there are differences among species in the degree of sensibility to white light. *Ciona* is much less sensitive to light than is *Mya* or *Pholas.* *Mya* requires about 5 meter-candle-seconds as a minimum amount of energy for stimulation, while *Ciona* needs nearly a thousand times as much (Hecht, 1919–20). This may perhaps be due merely to differences in concentration of the sensitive material. However, it can also be shown that the animals differ strikingly in their sensibility to different parts of the spectrum, facts which are simply explained by assuming differences in the absorption spectra of the sensitive materials concerned with receiving the light energy.

Ciona is comparatively so insensitive to light that it has not been possible to measure the effectiveness of different parts of the spectrum with any greater fineness than will enable one to say that the middle of the visible region is the most effective. *Mya*, however, has been measured in detail (Hecht, 1920–21, *b*), and the data show a well defined maximum of sensibility at 500 mμ, a secondary region of high sensibility at 570 mμ, and a rapid decline of sensibility on either side of the peaks. In the present paper it is proposed to record the measurements made in similar fashion with the Mediterranean lamellibranch *Pholas dactylus*, and to compare them with those of *Mya* and with other animals.

II.

The principle of the measurements is to isolate different portions of the visible spectrum by means of filters, and to determine the amount of energy required for each part in order to elicit the *same sensory effect* in the animal. Technically the experiments involve

three parts. First it is necessary to determine the energy distribution in the spectrum of the lamp and the transmission of the filters in order to know the energy content and the composition of the light transmitted by each filter. Second, it is required to vary this energy in a known manner so as to subject a given animal to different amounts of it. And third, it is necessary to measure the responses of the animal in order to determine when the sensory effects of different portions of the spectrum are identical.

The source of light used in these experiments was a 1000 watt Phillips lamp of the concentrated-filament, half-watt-per-candle variety run at a constant voltage of 200 volts. Its energy distribution

TABLE I.

Energy Distribution in the Spectrum of a 1000 Watt Concentrated Filament Phillips Lamp Running at 200 Volts. The Energy at 550 mμ is Placed at Unity.

λ	Energy
mμ	
450	0.34
500	0.63
550	1.00
600	1.41
650	1.82
700	2.21

was determined at the Physikalisch-Technische Reichsanstalt in Berlin by Dr. W. Dziobek, whom it is a pleasure to thank for his kindness in making these and other measurements for me. The data are given in Table I. The filters were Wratten Filters 72, 73, 74, 75, and 76 of the "monochromatic" series made by the Eastman Kodak Company. The transmissions of these filters are well known (*cf.* Hecht, 1920–21, *b*), and are to be found in detail in a booklet—Wratten Light Filters—published by the Eastman Kodak Company. The energy of the lamp at any wave-length, as taken from Table I, and multiplied by the transmission of a filter at that wave-length gives the amount of energy of that frequency transmitted by the filter. These calculations were made for each filter for every 10mμ of its transmission.

The energy transmission was then plotted on a large scale for each filter, and the total energy transmitted by each filter determined by measuring the area included under the transmission curve. The center of gravity of each area was then found; this corresponds to the wave-length on either side of which an equal amount of energy is transmitted by the filter. Table II gives the resulting information. It contains the number of the filter, its central wave-length, to the nearest 5 mμ, and the total amount of energy transmitted by it in series with the 1000 watt lamp.

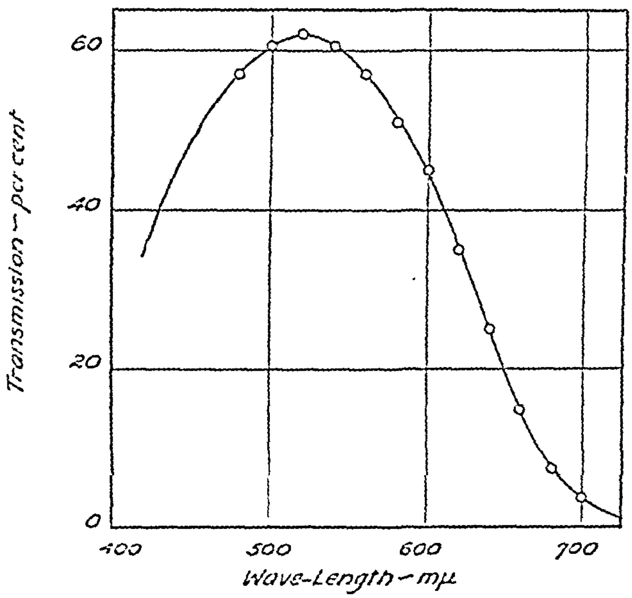

FIG. 1. Transmission spectrum of the copper chloride solution used in Series IV.

In one series of experiments I used an additional filter in conjunction with those already mentioned. This consisted of a layer, 3.7 cm. thick, of 1 per cent aqueous solution of $CuCl_2 \cdot 2H_2O$, whose purpose was to exclude the near infra-red. The transmission of this filter is given in Fig. 1, and is derived from measurements made by Dr. Dziobek for me. This filter was used in Series IV with each of the Wratten filters. Its influence on the composition of the light transmitted is determined by multiplying the energy of the lamp at a given wave-length by the transmission of the copper chloride at that wave-length, and by the transmission of the particular Wratten filter at the same

point. The product gives the energy transmitted at that wave-length. As before, the computations were made at 10 mμ intervals, the curves plotted, the areas under them measured, and the central wave-lengths determined. The results are given in Table II. This table gives the information required by the first part of the procedure, namely, the energy content and composition of the light transmitted by the different portions of the spectrum used for experimentation.

The second part, that of varying the intensity of these parts of the spectrum was accomplished (*a*) by keeping the animals at different distances from the light source and calculating the relative intensities on the inverse square law; and (*b*) by interposing neutral filters, made

TABLE II.

Central Wave-Length and Relative Energy Content of Light from 1000 Watt Lamp Transmitted by Wratten Filters.

Filter No.	Central wave-length	Relative energy content	
		Series I, II, III	Series IV
	$m\mu$		
76	450	100	100
75	485	275	339
74	535	113	156
73	585	260	316
72	615	104	82

of fogged photographic plate, which were calibrated photometrically and spectrophotometrically. In this way an animal could be subjected to any desired intensity of light of known spectral composition.

The third part of the procedure consisted in exposing animals to these various lights, measuring their response, and determining at what intensities the differently colored lights produce the same sensory effect. The reaction time of *Pholas* to light varies inversely with the intensity of the light. With a given part of the spectrum it is thus possible by varying its intensity (I) and measuring the reaction time (r) of an animal to construct a curve relating I and r at that wave-length. Such a curve can be determined for the five different parts of the spectrum isolated by the Wratten filters. From the family of

curves obtained in this manner there can then be found the energy required by each monochromatic patch of the spectrum to cause a response after the same reaction time. The relation among the energies of the different parts of the spectrum gives the relative effectiveness of the various parts in the photosensory stimulation of *Pholas*.

III.

The actual procedure with the animals was as follows. A number of animals were completely dark-adapted by being kept in the dark for about a week. Each animal remained in its own rectangular dish in a light-tight water bath whose average temperature was 16.7°C. An animal was removed from the thermostat, placed on the experimental bench at a definite distance from the light source, exposed to the selected light, its reaction time measured, and at once returned to the thermostat. Half hour later it was again taken out, and placed at another distance, its reaction time measured, and again returned. Half hour later this was repeated at still another distance. At least three intensities were used so as to get the relation between intensity and reaction time for a particular portion of the spectrum. The filter combination was then changed to secure a different part of the spectrum, and the reaction time of the animal was measured at half-hour intervals at three different intensities. This was continued until the five selected parts of the spectrum had been investigated for a given animal.

In this manner I ran four series of experiments. Series I had 4 animals; Series II had 6 animals; Series III had 7 animals. Each of these series was carried through in one day. As a source of light there was used the Phillips lamp and the Wratten filters. Several weeks after these experiments, I ran Series IV which differed in three respects from the other three series. First, it consisted of a larger number of animals, 15 to be precise. Second, it contained the copper chloride filter in addition to the Wratten filters. And third, the measurements were made one day, and on the following they were repeated, but in the inverse order of spectral parts and of intensities.

The data secured in the four series of experiments are presented in Table III. From mere inspection of the table it is apparent that the sensibility distribution along the spectrum is pretty much the same

in all four series, and that the maximum sensibility lies at about 535 mμ. However, a closer examination of the data is necessary in order to determine accurately the relative sensibility of the animals to the different wave-lengths. This treatment of the data will be given in detail for Series I; the method for the other series is identical, and only their results will be given.

TABLE III.

Relation between Intensity of Illumination and Reaction Time of Pholas to Light of Different Wave-Length. Reaction Time, r, in Hundredths of a Minute.

Wave-length	Series I		Series II		Series III		Series IV	
	I	*r*	*I*	*r*	*I*	*r*	*I*	*r*
mμ		*hm.*		*hm.*		*hm.*		*hm.*
	347.0	2.70	141.0	2.90	347.0	2.63	174.0	2.79
	77.6	3.33	77.6	3.37	42.7	3.87	28.2	3.63
450	57.5	3.48	22.9	4.20	5.13	5.91	11.8	4.79
	22.9	4.28	9.55	4.80				
	9.55	6.18						
	63.1	3.68	63.1	3.03	63.1	3.11	95.5	2.94
485	30.2	4.25	26.3	3.52	26.3	4.00	39.8	3.57
	14.1	5.58	14.1	4.88	14.1	4.57	21.4	4.30
	25.7	3.48	87.1	2.60	25.7	3.03	43.7	3.04
535	10.7	4.00	25.7	3.12	10.7	3.59	18.2	3.62
	5.75	5.40	10.7	4.00	5.75	4.21	9.77	4.34
			5.75	4.47	3.16	5.46		
	372.0	3.40	372.0	2.73	201.0	2.96	302.0	3.13
585	151.0	3 95	25.1	4 12	60 3	3.82	89.1	3.83
	60.3	5.20	13.5	6.40	25.1	5.03	37.2	4.87
	912.0	3.58	912.0	3.27	363.0	3.73	347.0	3.63
615	363.0	4.28	363.0	3.67	148.0	4.44	141.0	4.35
	148.0	6.40	148 0	3.95	81 3	4.94	77.6	5.38

Fig. 2 gives the data of Series I in graphic form. It is apparent that the curves describing the relation between log *I* and the reaction time, *r* for the different wave-lengths are parallel to one another. In order to find the relative amounts of energy required by the different wave-lengths to produce the same sensory effect, one can read off

from the plot the abscissa values corresponding to a given ordinate value. The reciprocals of these I values will then give the relative stimulating capacities of the different parts of the spectrum. Such measurements of the intensity may be made at convenient values of the ordinates, say at 6.0, 5.0, etc., the results averaged, and the reciprocals taken. Simpler still is to make the measurements for the curve as a whole by determining the logarithmic distance on the abscissas through which the curves have to be moved to the left in order to coincide with the one corresponding to the most effective part of the spectrum. This amounts to finding the factor by which the

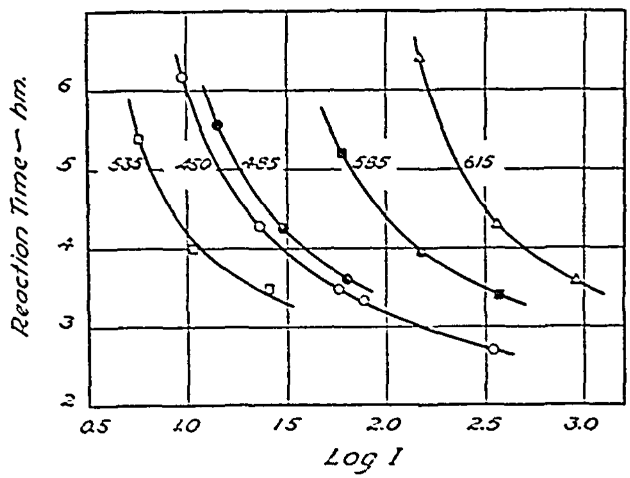

FIG. 2. Relation between log I and the reaction time of *Pholas* to light from the different portions of the spectrum as indicated by their central wave-length. Data of Series I. Reaction time is in hundredths of a minute.

intensity values of the points on a given curve must be multiplied in order to fit on the curve farthest to the left. The factors so found are equal to the ratios of the intensity of the most effective wave-length to each of the intensities of the other wave-lengths required to produce the same sensory effect. If I_{max} is the intensity of light at 535 mμ necessary to produce a given reaction time, and I_λ the intensity necessary to produce the same effect at any other value of λ, then the factor by which the I_λ values of any curve in Fig. 2 must be multiplied in order to coincide with the curve at the extreme left, $\lambda_{max} = 535$, will be equal to I_{max}/I_λ. Obviously the ratio I_{max}/I_λ gives the relative

stimulating capacity of the different parts of the spectrum. Fig. 3 shows the five curves of Fig. 2 whose intensities have been multiplied by the proper factors, and superimposed one on the other. The adequacy of this method may be judged by the way the points for the different wave-lengths lie on the common curve. Similarly in Fig. 3 are given the composite curves for the other three series of experiments.

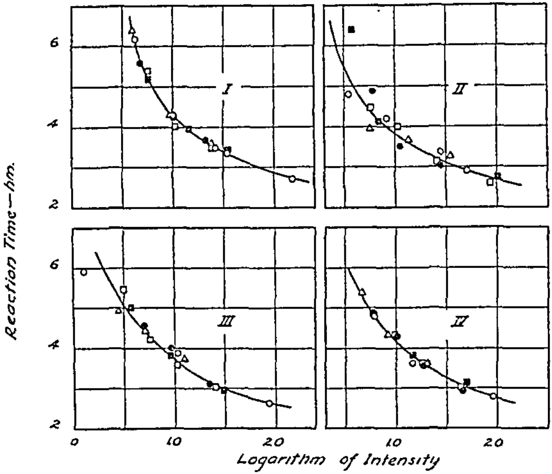

Fig. 3. Data of four series of experiments relating log I and reaction time of *Pholas* to lights of different wave-length. The symbols for the different points are the same as in Fig. 2.

Table IV gives the factors which have been used to superimpose the various curves of the four series of data, and which correspond to the ratio I_{max}/I_{λ} for each. It is apparent that the relative stimulating capacities of the different parts of the spectrum are similar for the different series. Fig. 4 shows the data of Table IV graphically. From the smooth curves drawn, it is apparent that 550 mμ is the most effective portion of the spectrum. On the red side the effectiveness drops

very rapidly to almost nothing. It is for this reason that it is possible to make all of the manipulations with *Pholas* by the dim light of a 10 watt ruby lamp; the animals are insensitive to such illumination. On the blue side of the maximum the effectiveness drops rapidly, but only to about half the maximum, and then apparently rises again. Series II and III do not actually indicate this rise. But Series I and IV show it clearly. Series IV is undoubtedly the best of the experiments; it is based on about as many animals as all the rest together, and on two readings for each animal for each point. The rise toward the violet end is therefore probably a real phenomenon, and would indicate that the animals may be sensitive to the near ultra-

TABLE IV.

Relative Effectiveness of Different Wave-Lengths in Stimulating Pholas. The Figures in the Table Give the Values of the Factor a × 100 for the Different Wave-Lengths and Different Series of Table III.

Series	Relative effectiveness				
	450 $m\mu$	485 $m\mu$	535 $m\mu$	585 $m\mu$	615 $m\mu$
I	44.7	33.9	100	9.55	2.69
II	36.3	42.7	100	27.5	3.80
III	25.1	35.5	100	15.1	3.47
IV	52.5	47.9	100	16.2	5.89

violet. Unfortunately these computations were made after leaving Naples, and this possibility has not been tested.

IV.

The relative effectiveness of the visible spectrum in stimulating *Pholas* is apparent from Table IV and Fig. 4. It is very unlikely that this variation in effectiveness is due to any differences in the properties of the different parts of the spectrum. The frequencies represented are too near one another to suppose any real differences in photochemical characteristics. The variations shown in Fig. 4 are thus most probably descriptive of something in the organism. The effect produced on *Pholas* by the different parts of the spectrum is a qualitatively invariable reflex. The derivation of the data in

Table IV and Fig. 4 depends on a comparison of quantitatively identical effects in this qualitatively invariable reflex. One may assume that the production of these identical effects depends on the reception of a given amount of energy, regardless of wave-length, by a sensitive material in the sense cells of *Pholas*, and its conversion into the photochemical action which starts the photoreceptor process. If this assumption is correct, then the curves in Fig. 4 represent the absorption spectrum of the photosensitive substance in *Pholas* plus any screening pigments that may be intimately associated with it.

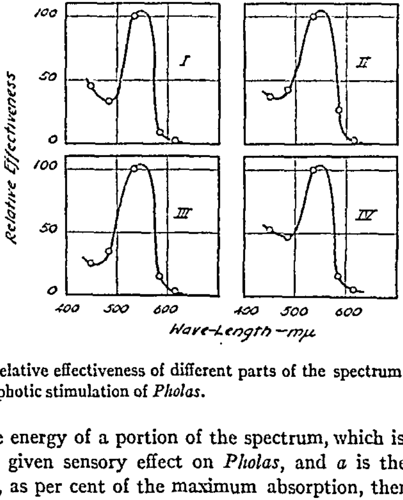

FIG. 4. Relative effectiveness of different parts of the spectrum in eliciting a response to photic stimulation of *Pholas*.

If I_λ is the energy of a portion of the spectrum, which is required to produce a given sensory effect on *Pholas*, and a is the absorption coefficient, as per cent of the maximum absorption, then aI_λ will be the amount of the energy at that wave-length which is absorbed by the photosensitive system. But the absorbed energy necessary to produce a constant sensory effect has been assumed constant. Call this energy K. Then $aI_\lambda = K$, and $a = K/I_\lambda$. In other words the absorption coefficient of the photosensitive system is proportional to the reciprocal of the energy required at a given wave-length to produce a constant sensory effect. These reciprocals are of course

the values in Table IV and Fig. 4. The curves may be then regarded as expressing the absorption spectrum of the photosensitive system in *Pholas*, and as a specific characteristic of this animal.

Fig. 5 shows a comparison between the effectiveness of the spectum for *Mya*[1] and *Pholas*. Considered as indirectly determined absorption spectra, these curves show that the photosensitive systems in these two animals are quite different. Thus although the two animals possess an extraordinary similarity in the general characteristics and organization of their photosensory process, the materials of which these systems are composed are very likely different.

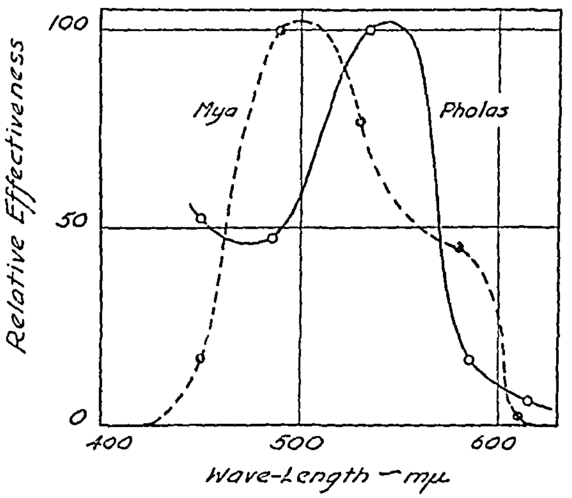

FIG. 5. A comparison of the relative effectiveness of the spectrum in the stimulation of the lamellibranchs, *Pholas* and *Mya*. The curve for *Pholas* is from Series IV.

The conclusion that different animals possess different photosensitive materials would seem too self-evident, were it not for the fact that Hess (1910) has emphasized their similarities to the extent that he supposes the effectiveness of the spectrum to coincide with the luminosity curve of the totally color-blind human eye. The number of

[1] The data are taken from Table II and Fig. 5 of the paper on *Mya* (Hecht, 1920–21, *b*). Due to a typographical error the decimal point in the values for Filters 73 and 74 in Table II of this paper was put one place too far to the right. The correct values are, *e.g.* 2.18 and 1.29 respectively, and are correctly given in Fig. 5 of the same paper.

careful measurements which have appeared in recent years in this connection (Laurens and Hooker (1920), Loeb and Wasteneys (1916), Mast (1917), Hecht (1920–21, *b*), Crozier (1923–24))leave no doubt however that such a generalization is an inadequate expression of the data as known. Special attention is here directed to the differences between *Mya* and *Pholas* because of the very striking similarities shown in the other characteristics of their photosensory processes.

V.

Although these data on the effectiveness of the spectrum show how different are the absorption spectra of the photosensitive systems of *Mya* and *Pholas*, they show, at the same time, how fundamentally similar are the organization and photochemistry of the two systems. This will become evident in the present section.

Examination of the material in Figs. 2 and 3, relating intensity and reaction time, shows that curves have a consistently uniform appearance regardless of wave-length or series. It is significant to inquire into the form of this relation between intensity and reaction time, and to determine its precise meaning in terms of our knowledge of the photosensory process.

It was shown for *Mya* (Hecht, 1920–21, *a*) and for *Ciona* as well (Hecht, 1925–28) that if the intensity, I, of the stimulating light is kept constant, and the time (t) of its action varied, the photochemical effect (E) is very nearly a linear function of the time. In other words

$$E = k_1 t \tag{1}$$

where k_1 is a constant. Similarly if the time of action is constant and the intensity varies, then the effect is proportional to the logarithm of the intensity. This may be written as

$$E = k_2 \log I \tag{2}$$

where k_2 is a constant. It therefore follows from these two equations that

$$E = k\, t \log I \tag{3}$$

and experiments with *Mya* showed that when I and t are both varied, equation (3) holds true experimentally. It is significant that equation (3) is valid for the photosensory responses of *Pholas*.

Perhaps the simplest way of demonstrating this is as follows. The reaction time (r) is composed of two parts, the exposure period (t) and the latent period (p), so that

$$r = t + p$$

and

$$t = r - p. \tag{4}$$

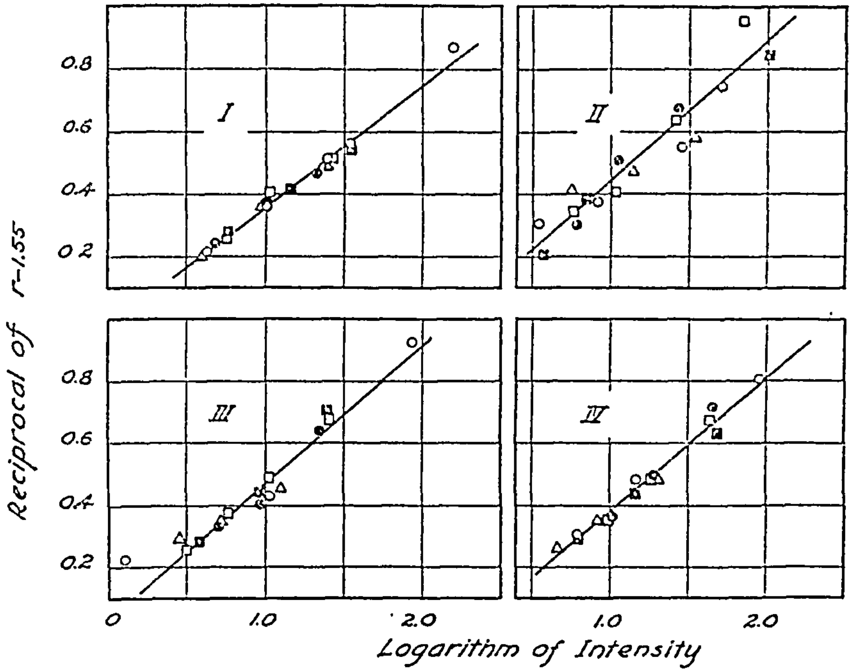

FIG. 6. Relation between log I and the reciprocal of $r - p$, where r is the reaction time and p the latent period.

This value of the time of action of the light may now be substituted in equation (3) which then becomes

$$E = k\,(r - p) \log I$$

and

$$\frac{E}{r - p} = k \log I. \tag{5}$$

It is apparent that since E is constant the reciprocal of $(r - p)$ plotted against log I should give a straight line if equation (3) is true.

Fig. 6 shows the relation between the reciprocal of $(r - p)$ and $\log I$ for the four series of experiments made with *Pholas*. It is assumed that $p = 1.55$ hm., no measurements of the latent period having been made with these particular animals. This value of p is very likely correct, because a value of $p = 1.67$ hm. at 16.5°C. was actually obtained with 16 animals in a series of experiments on the dark adaptation of *Pholas* made within a few days of Series IV of the present experiments (Hecht, 1926–27; Table II). In making Fig. 6, the composite data of Fig. 3 were used, these in turn having been derived from Tables III and IV. The straight lines in Fig. 6 show that equation (5) describes these data, and therefore that the relationships expressed by equations (1) and (2) which enter into the derivation of equations (3) and (5) are applicable not only to the photosensory process in *Mya* and *Ciona* but in *Pholas* as well. The present experiments have thus shown that the photosensory systems of these three animals are specifically different though they are fundamentally the same.

SUMMARY.

The most effective point in the visible spectrum for the stimulation of *Pholas* is 550 mμ. On the red side, the effectiveness drops rapidly to almost zero. On the violet side, the effectiveness drops to about half, and rises again in such a way as to indicate a possible second maximum in the near ultra-violet.

On the basis of certain ideas these data are assumed to represent the properties of the absorption spectrum of the photosensitive system in *Pholas*. A comparison with *Mya* shows that the absorption spectra of the photosensitive systems in the animals are distinctly different.

Nevertheless the way in which intensity and reaction time are related in the two animals are found to be identical. The conclusion is then drawn from this and from previous work, that although the fundamental properties of the photoreceptor process show an identical organization in several different animals, the materials which compose these processes are specific.

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POSITIVE AND NEGATIVE CURRENTS OF INJURY IN RELATION TO PROTOPLASMIC STRUCTURE.*

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Much may be learned about the nature of protoplasm by studying its transformations during the process of death. Such alterations have been observed by a method which has marked advantages since it permits us to follow very rapid changes which are often of primary importance. It may be added that certain complications inherent in tissues have been avoided by using single multinucleate cells.

The outcome, which supports the idea that protoplasm is made up of layers differing considerably in their properties, appears to necessitate a change in the traditional view that the current of injury is negative[1] for the observations show that it can be rendered positive or negative according to the will of the experimenter.

The experiments described in this paper were performed on single multinucleate cells of *Nitella flexilis*[2] according to the methods given in a previous paper[3] (which contains a description of the technique and of the apparatus). A flowing contact was used in most cases. Especial care was taken to employ only normal cells, and to avoid injury (except in applying toxic agents). The current of injury was produced by chloroform which has previously been found useful for experi-

* The writers desire to express their gratitude to the Carnegie Institution of Washington, D. C., which generously provided for the beginning of these researches in 1922–25.

[1] A discussion of the literature will be deferred to a later paper dealing with cell groups and tissues. It may be mentioned that a few experiments on the effect of chloroform on *Nitella* were performed by Haacke (Haacke, O., *Flora*, 1892, lxxv, 455). Hörmann (Hörmann, G., Studien über die Protoplasmaströmung bei den Characeen, Jena, 1898) states that stimulation of one end of a *Nitella* cell produces a wave of negativity.

[2] Kindly identified by Dr. J. S. Karling.

[3] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391.

ments on injury.[4] The experiments were carried out at room temperature, ranging from 20° to 25°C. but the variation in any one experiment was less than 1°C.

The procedure may be illustrated by an experiment with 0.05 M KCl[5] in contact with the cell at *A* and *C* (as shown in Fig. 1). Fig.

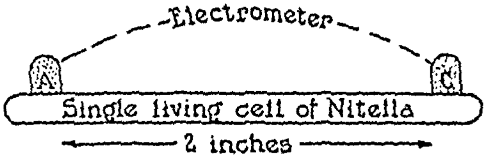

FIG. 1. Diagram to show the arrangement of the experiments. In most cases flowing contacts are employed at *A* and *C*. In some cases the ends of the cell dipped into two cups hollowed out of a block of paraffin: in experiments with sap cotton soaked in sap is applied. When necessary (with more concentrated solutions) cotton soaked in distilled water is applied for a short stretch between *A* and *C* but a space is left on each side of the cotton to prevent short circuiting.

2 shows at the start a small potential difference[6] between *A* and *C*.[7] We now substitute for 0.05 M KCl at *C* a solution of 0.05 M KCl saturated with chloroform. This injures the protoplasm at *C* and produces characteristic changes in the curve but it subsequently reaches a steady state which is assumed to mean that the protoplasm

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 709. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391. Chloroform is soluble to the extent of about 0.4 per cent by volume (about 0.6 per cent by weight) and hence does not noticeably affect the osmotic pressure or concentration of electrolytes.

[5] This gives practically the same result as sap or artificial sap. It would appear that this result must be due primarily to the effect of the cations since the halide content of sap is about the same as that of 0.1 M KCl, but the K content of sap is much less.

[6] To avoid misunderstanding we may state that we use the term electromotive force to designate the force present at an electrode surface: if we tap off at two points in the circuit we obtain a potential difference which depends on the resistances involved.

[7] The recorded values would probably be a trifle higher if we could eliminate short-circuiting effects in the cell (Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83). Diffusion potentials are small enough to be neglected (in view of the concentration of KCl present).

at C is dead.[8] For reasons given in a previous paper[3] we now regard the potential difference as due entirely to the living protoplasm at A. We now apply 0.05 M KCl saturated with chloroform to A and obtain a curve similar to that already observed except that the signs are reversed. This is to be expected since the curve always records the state of A with reference to C and any changes occurring at C will have their signs reversed: hence to determine the effect of chloroform on the protoplasm we must take the latter part of the curve (when the chloroform is applied to A) where the signs have their true value (or we may take the first part of the curve and reverse the signs).

We see that when A is injured by applying chloroform dissolved in 0.05 M KCl A becomes more positive[9] with reference to C. Since the

[8] The use of a flowing contact[3] largely eliminates disturbances due to the coming out of sap immediately after the death of C. If the flowing contact were not employed we should not have the same salt solutions in the cell wall at A and C and in consequence there might be a potential difference (sap coming out at C earlier than at A would tend to make the cell wall at C more negative so that on the record A would appear more positive). The flowing contact promptly washes the sap out of the cell wall and thus removes this source of disturbance. As an example of this we may consider the following experiment: Live cells were placed in pure chloroform (not in an aqueous solution of chloroform) which quickly kills them without allowing salts to escape from the cells. One end of the cell was then cut and the protoplasm and sap squeezed out for a distance of an inch so that the lumen was filled with air and could not supply salts to the cell wall any more than the living protoplasm would. We will call this end A and the other C. Both A and C were alike in having the cell wall imbibed with sap, due to the death of the protoplasm (which is not quite the same thing as applying sap from the outside, as will be discussed in a later paper), but they differ in that C has a reservoir of sap in the lumen of the cell which A has not. On leading off from A to C, with 0.05 M KCl applied to each, there was practically no potential difference but when 0.001 M KCl (flowing junction) was applied to A, it quickly became positive showing that the salts were being rapidly washed out. When the value became constant, 0.001 M KCl (flowing junction) was applied at C and the potential difference rapidly fell to zero showing that the salts were washed out of the cell wall despite the reservoir of sap in the lumen of the cell.

The flowing contact did not produce complications except possibly in very dilute solutions and if disturbances occurred in experiments of any sort (as shown by the fact that the curve did not fall to zero when the cell was killed) the experiments were rejected.

[9] In all cases we obtain essentially the same result when we apply a toxic agent at A whether we have previously killed C or not. The only difference is that if C

protoplasm at *C* is dead we may regard its electromotive force as approximately zero[3] so that all the potential differences observed are

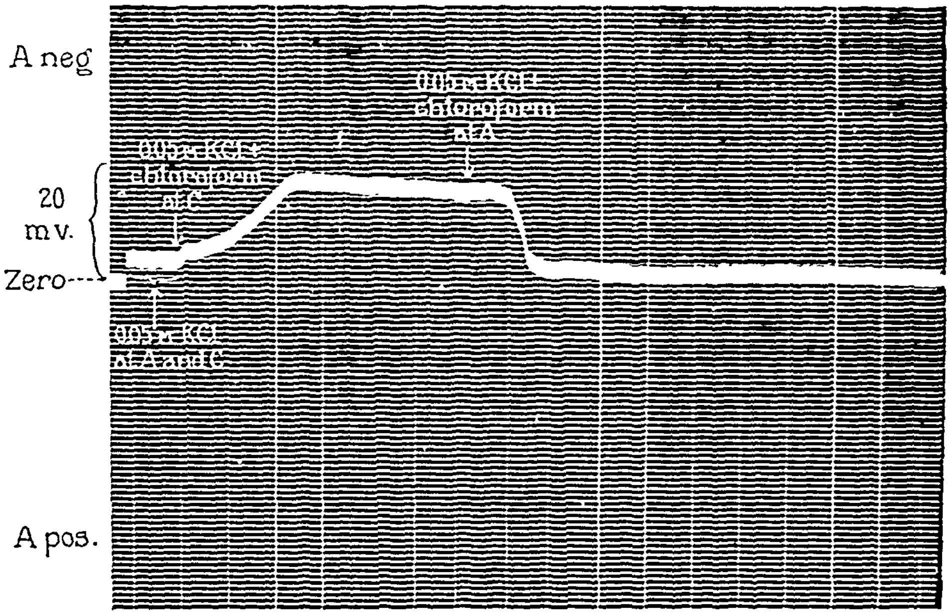

FIG. 2. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.05 M KCl at *A* and *C*. When 0.05 M KCl saturated with chloroform is applied at *C* the curve (which records the potential difference of *A* with reference to *C*) shows that *A* appears to become more negative but since it is only *C* which changes, the curve really shows that *C* is becoming more positive. The potential difference between *A* and *C* soon reaches a fixed value which is practically all due to the living protoplasm at *A*. A solution of 0.05 M KCl saturated with chloroform is then applied at *A*, giving a death curve which resembles that already observed but with opposite signs since the signs now have their true value (*C* being dead remains constant). The curve shows that under these conditions injury causes the protoplasm to become more positive after which the potential difference approaches zero.

The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

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remains alive it adds a certain constant (positive or negative) value (due to the presence of the living protoplasm at *C*): in consequence the curve of *A* does not approach zero at the end but reaches a steady state at a definite positive or negative value which represents the potential difference across the protoplasm at *C*.

due to A which, as shown in a previous paper,[3] can apparently remain normal for some time after the death of C.

It will be noticed that there is no trace of the traditional negative current of injury. The observed change in potential difference caused by the toxic agent is wholly in the positive direction.

It may simplify matters if before describing other experiments we present an hypothesis which brings the observed phenomena under a single point of view and which may thus facilitate the subsequent treatment.

According to this hypothesis the protoplasm has an outer (X) and an inner (Y) surface layer, both of which are probably non-aqueous,

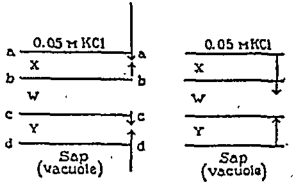

FIG. 3 *a*. FIG. 3 *b*.

FIG. 3. Hypothetical diagram to illustrate the condition of the protoplasm in contact with 0.05 M KCl. The direction in which the positive current tends to flow is shown by the direction of the arrows, the relative magnitude of the electromotive force being indicated by their length. Fig. 3 *a* represents a possible conception of the potential differences; Fig. 3 *b* presents the same thing in simplified form. The potential difference across the protoplasm is said to be negative since the negative arrow at X is longer and the positive current tends to flow as indicated in Fig. 4 *b*.

with an aqueous layer, W, between them. On this basis we may diagram the potential differences in the protoplasm in the manner shown in Fig. 3 *a* and 3 *b*, the arrows representing the direction in which the positive current tends to flow and their length indicating the relative magnitude of the potential difference.[10] The underlying theory of these effects has been discussed in a previous paper[11] and

[10] The arrows are hypothetical illustrations and their lengths can be varied accordingly.

[11] Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83.

the conclusion reached that in general the cations have a greater tendency to enter X and Y than the anions. Hence we may suppose that at the surfaces a, b, c and d (Fig. 3 a) the potential differences may be represented by the arrows if they are due to phase boundary potentials. Since we are merely concerned with the resultant of these

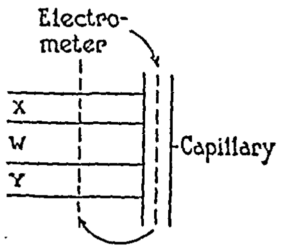

FIG. 4 *a*.

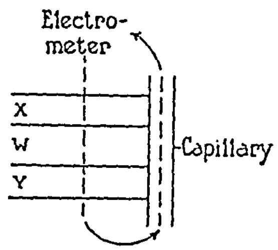

FIG. 4 *b*.

FIG. 4 *a*. Diagram of a circuit through the protoplasm at a single spot where an imaginary capillary has been inserted so as to lead off from the outside of the protoplasm to the inside. In this case the potential difference across the protoplasm is said to be positive because the electrometer shows X to be positive to Y.

FIG. 4 *b*. As in Fig. 4 *a* but in this case the potential difference across the protoplasm is said to be negative because the electrometer shows X to be negative to Y.

effects it is more convenient to give the diagram the simpler form shown in Fig. 3 *b* in which only two arrows[12] are employed: this would also be appropriate if diffusion potentials[13] play a part.[14]

In the diagram in Fig. 3 *b* the arrow at X is longer than that at Y indicating that the positive current tends to flow as in Fig. 4 *b*. Hence

[12] The actual length of the arrows is not significant: it is merely intended to show that the X arrow is longer than the Y arrow.

[13] Such diffusion potentials might well be due to organic substances produced by the cell (*e.g.*, in the layer W). It is commonly said that diffusion potentials cannot produce potential differences of the magnitude found in living cells. This would be true of aqueous solutions but in non-aqueous media or in such structures as the collodion membranes studied by Michaelis and his coworkers higher values may be possible.

[14] Whether we suppose that the potential difference results chiefly from diffusion potentials due to substances produced in W or to phase boundary potentials (following the scheme in Fig. 3 *a*) it seems highly probable that the arrows in Fig. 3 *b* should point in opposite directions.

if we arrange an experiment as in Fig. 1 with 0.05 M KCl at A and C and lead off from A to C after C has been killed by chloroform (thus reducing its potential difference approximately to zero) we should expect the positive current to flow from C to A so that A would appear negative. This is actually the case, as is evident from Fig. 2.

Although little is known regarding electrical conditions in the protoplasm it may be of interest to consider certain possibilities which would exist if the cell conformed to the diagram in Fig. 5. Here we confine ourselves to the assumption that there is but one seat of electromotive force in X and one in Y (but they may exist at both surfaces of any

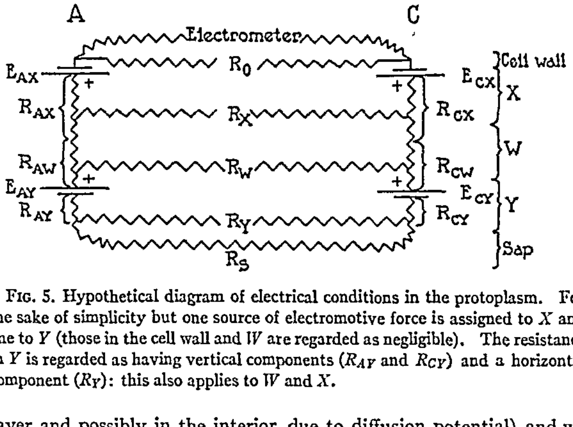

FIG. 5. Hypothetical diagram of electrical conditions in the protoplasm. For the sake of simplicity but one source of electromotive force is assigned to X and one to Y (those in the cell wall and W are regarded as negligible). The resistance in Y is regarded as having vertical components (R_{AY} and R_{CY}) and a horizontal component (R_Y): this also applies to W and X.

layer and possibly in the interior, due to diffusion potential) and we have arbitrarily located this at the outer surface in each case. On this basis the total electromotive force may be regarded as $E_{AY} - E_{AX} - E_{CY} + E_{CX}$.

The resistance in each layer may be regarded as consisting of a vertical component (such as R_{AY}) and a horizontal component (such as R_Y). The resistances R_X and R_Y may be relatively high: R_W and R_S are probably less. As to the resistance R_O it may be said that the measurements of Dr. Blinks show that when we cut out a portion of a

cell about an inch in length, remove the protoplasm and sap by squeezing, rinse in tap water, squeeze the contents out and dry the surface lightly with filter paper, the resistance is in the neighborhood of a megohm. That of a similar length of intact cell is about half as much which might indicate that under these conditions the resistance of the living protoplasm and sap taken together is not far from that of the cell wall.[15]

But, according to Dr. Blinks, it seems possible that the resistance due to the living protoplasm and sap taken together (which we may call R_P) may be largely due to polarization: hence if measured with currents as small as those present in our ordinary experiments R_P might be quite small in comparison with R_O. In that case we might write

$$\text{observed P.D.} = \text{E.M.F. in circuit} \left(\frac{R_O}{R_O + R_P}\right)$$

and the value of $\frac{R_O}{R_O + R_P}$ would be not far from 1 so that the measurements described in this paper would not be far from the true values of the electromotive forces in the circuit. That this is actually the case is indicated by the measurements obtained when a capillary is inserted in the manner employed for *Valonia*.[11]

Let us now consider what would happen if we had a simpler system from which the layers X and W were omitted. We should then be measuring by means of the electrometer the potential difference across the ends of R_O and we should have

$$\text{observed P.D.} = E_{AY} - E_{CY} \left(\frac{R_O}{R_O + R_{AY} + R_{CY} + R_{YS}}\right)$$

in which R_{YS} is the combined resistance of R_Y and R_S (and is equal to $(R_Y)\ (R_S) \div R_Y + R_S$).

The application of chloroform to C would presumably do away

[15] If we call the combined resistance of the living protoplasm and the sap R_P we might employ the usual equation for resistances in parallel and regard the effective resistance of the living cell as equal to $(R_P)\ (R_O) \div R_P + R_O$. If we have $R_P = R_O = 1$ megohm the effective resistance will be equal to 0.5 megohm.

with E_{CY} and reduce R_{CY} to negligible proportions so that the remaining potential difference would be

$$\text{observed P.D.} = E_{AY}\left(\frac{R_O}{R_O + R_{AY} + R_{YS}}\right).$$

When we speak of the potential difference across the protoplasm at A[16] (after C has been killed) it seems by no means impossible that we must understand it as conforming to a scheme which is similar to this but one which involves all the electromotive forces and resistances in the cell (including any possible eddy currents, as discussed in a previous paper[11]).

Future investigation must decide to what extent the conditions in the protoplasm are represented by such a scheme as that shown in Fig. 5. It is, of course, evident that the cell wall, X, W and Y are not actually insulated from each other as they are in the diagram nor are the electromotive forces confined to A and C.

We may suppose that if we have a simple system such as is represented by the diagram in Fig. 5 the electromotive force after the killing of C is

$$\text{E.M.F.} = E_{AY} - E_{AX}$$

of which we measure by means of the electrometer only a certain fraction so that we may write

$$\text{observed P.D.} = n(E_{AY} - E_{AX}) = n(E_{AY}) - n(E_{AX}).$$

If we put $y = n(E_{AY})$ and $x = n(E_{AX})$ we may write

$$\text{observed P.D.} = y - x.$$

[16] The observed P.D. does not show much change when we shorten the distance between A and C (both being intact) so that we may regard the observed potential difference as practically that of the area directly under A. This fact indicates that R_O cannot be small in comparison with $R_{AY} + R_{CY}$ for if it were the shortening of the distance between A and C would greatly diminish the observed potential difference since the value of $R_{AY} + R_{CY}$ would remain unchanged.

It is evident that we cannot arrive at the true value of the electromotive force at A (after killing C) unless we know the value of the fraction found by dividing R_O by the other resistances properly combined (measurements with an inserted capillary indicate that this value is not far from 1).

If we could destroy X without altering W or Y it is evident that both E_{AX} and R_{AX} would disappear and that this would not only change the electromotive force but would also alter the value of n. In view of this and possibly other complications (such as the presence

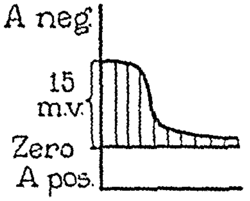

FIG. 6 *a*.

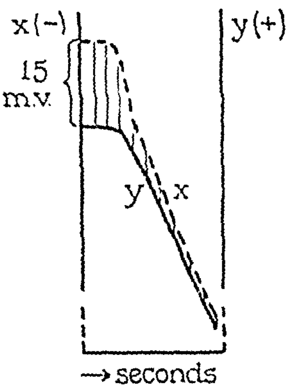

FIG. 6 *b*.

FIG. 6 *a*. Tracing of the latter part of the curve shown in Fig. 2 (after C has been killed by chloroform so that the entire electromotive force is due to the living protoplasm at A). The curve shows that at the start the value of A is negative (−15 millivolts). After the application of chloroform at A it becomes more positive since the potential difference diminishes and approaches zero. Certain ordinates are shaded for comparison with Fig. 6 *b*.

FIG. 6 *b*. Theoretical interpretation of Fig. 6 *a*. The ordinates as drawn represent the observed potential difference which is equal to that of Fig. 6 *a*. Fig. 6 *a* shows only the observed potential difference but Fig. 6 *b* indicates that this is equal to the difference between x with negative sign (as shown by the scale of ordinates at the left) and the value of y with positive sign (as shown by the scale of ordinates on the right). Each ordinate in Fig. 6 *b* has the same length as the one directly above it in Fig. 6 *a*, and the distance between x and y is equal to the distance from zero of the curve in Fig. 6 *a*. Since x and y are purely hypothetical curves they may be drawn in a variety of ways but the most natural assumption seems to be that they have a simple and regular form and they have been drawn in conformity with this idea.

Since we do not know the absolute value of the ordinates in Fig. 6 *b* but only the value of the difference between x and y (*i.e.*, the value of the ordinates as drawn) the scales of ordinates are interrupted toward the base by a dotted line to signify that below this point the ordinate extends for an indefinite distance. It should be noted that if the x and y curves come together the observed potential difference is zero but this zero has no relation to the absolute zero of Fig. 6 *b*.

of more sources of electromotive force, eddy currents,[11] etc.) we shall employ the equation P.D. $= y - x$ merely for convenience in discussion and only in a qualitative sense, meaning that in a general way the observed potential difference depends on a factor due to X and on one due to Y.

Let us now consider the mechanism of the death process. Since chloroform penetrates with extreme rapidity (often killing in a few seconds) and since the protoplasm is only a few microns thick it seems probable that Y will be affected almost as soon as X. If X and Y are in contact with the same solution (sap) or with solutions which have been found[17] to act alike (*e.g.* if X is in contact with 0.05 M KCl and Y in contact with sap) we may expect both to change at the same rate if both are equally sensitive. This is sometimes observed and we might in that case interpret the result in the manner shown in Fig. 6 *b*, since it seems reasonable to suppose that when chloroform acts on X and Y the values of x and y may fall off in regular fashion, as is observed in the death curves of tissues[18] and of single cells (as shown by the unpublished experiments of Dr. Blinks). This assumption is by no means necessary but we may adopt it as the most natural and as a matter of fact it suffices to explain all the observed curves. If, for example, the application of chloroform + 0.05 M KCl causes x and y to fall off in some such fashion as that shown in Fig. 6 *b* it is evident that if we plot the difference between them $(y - x)$[19] in the usual way we shall get the curve shown in Fig. 6 *a* which is a tracing of the latter part of the curve in Fig. 2.

We do not know the absolute value of the ordinates in Fig. 6 *b* but only the difference between them. Hence the scales of ordinates are interrupted toward the base by dotted lines to signify that below this point the ordinates extend for an indefinite distance. It should be noted that when the x and y curves come together, the observed potential difference (as in Figs. 2 and 6 *a*) is zero, but this zero has no relation to the absolute zero of Fig. 6 *b*.

The nature of the change produced by chloroform is uncertain. It

[17] This is found by applying sap at A and 0.05 M KCl at C when it is observed that both act alike in that the potential difference between A and C is zero. We obtain the same death curve with sap saturated with chloroform as with 0.05 M KCl saturated with chloroform.

[18] *Cf.* Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Philadelphia, 1922; *J. Gen. Physiol.*, 1922–23, v, 709. *Cf.* Klopp, J. W., *J. Gen. Physiol.*, 1924–25, vii, 39.

[19] Since the value of A is negative the ordinates of x (which is negative by convention) are made greater than those of y (which is positive by convention).

may cause a loss both of electromotive force and of resistance.[20] It is quite possible that the first effect of chloroform may be to produce in X and Y very minute openings or small electrical leaks (some of which may be almost instantly repaired). This might have a certain

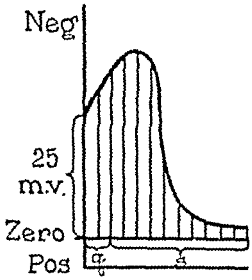

FIG. 7 *a*.

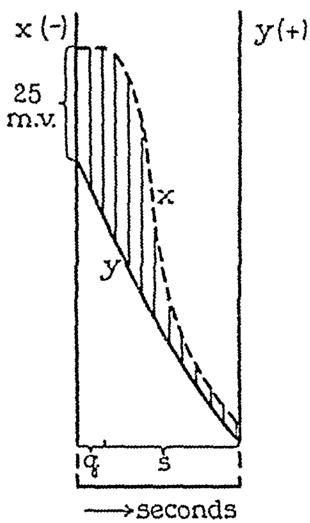

FIG. 7 *b*.

FIG. 7 *a*. Like Fig. 6 *a* but showing a different result. This is a tracing of the latter part of the curve in Fig. 8 (after the counter movement).

FIG. 7 *b*. Theoretical interpretation of Fig. 7 *a*. The ordinates as drawn show the observed potential difference (which is equal to that of Fig. 7 *a*). Fig. 7 *a* shows only the observed potential difference but Fig. 7 *b* indicates that this is equal to the difference between the value of x (with negative sign) and that of y (with positive sign). Each ordinate has the same length as the one directly above it in Fig. 7 *a*. During the period marked q the value of x remains stationary while that of y falls off corresponding to the fact that the curve in Fig. 7 *a* becomes more negative. During the period marked s the two curves in Fig. 7 *b* approach each other as the curve in Fig. 7 *a* approaches zero; if they should meet the potential difference would be zero (but this zero has no relation to the absolute zero of Fig. 7 *b*). *Cf.* Fig. 6 *b*.

similarity to the effects of mechanical injury. The fact that X and Y are changed so readily by chloroform and other lipoid solvents might suggest that they are lipoid: they are also very susceptible to

[20] In the case of *Laminaria* chloroform, ether, and alcohol cause a temporary increase of resistance (Osterhout, W. J. V., Injury, recovery, and death, Philadelphia, 1922) but this is not true of all organisms and may not be the case with *Nitella*. The increase of resistance is followed by a decrease which continues until death ensues: during this period recovery (partial or complete) is possible in the case of alcohol and to a very much smaller extent in the case of ether and chloroform.

the action of acid, alkali, concentrated salt solutions, and mechanical injury, but this might not be inconsistent with the assumption that they are lipoid in nature. We must also consider the possibility that they may act like proteins, agar, starch paste or kaolin.[21]

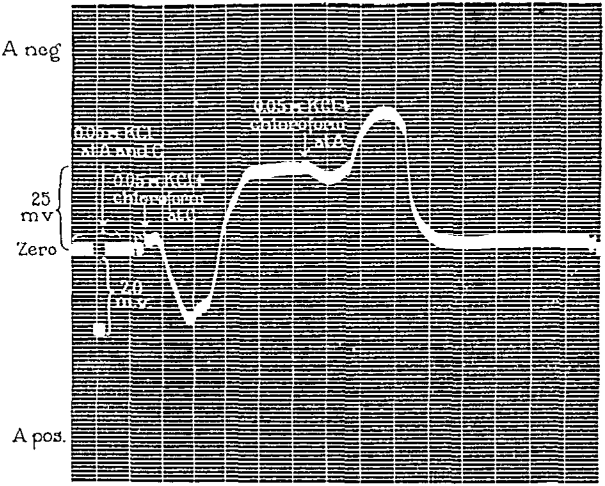

FIG. 8. Like Fig. 2 but showing a different result. After the counter movement following the application of chloroform at A (when the signs have their true value) we see that A becomes more negative, then more positive: the potential difference then sinks almost to zero. The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

The fact that alcohol produces effects similar to those of chloroform and that they are readily reversible up to a certain point suggests the possibility that the loss of potential difference may be due to an

[21] *Cf.* Hober, R., *Z. physik. Chem.*, 1924, cx, 142. Matsuo, T., *Arch. ges. Physiol.*, 1923, cc, 132. Mond, R., *Arch. ges. Physiol.*, 1924, cciii, 247. Fujita, A., *Biochem. Z.*, 1925, clxii, 245. Deutsch, W., *Arch. ges. Physiol.*, 1925, ccix, 675. Beutner, R., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 462.

increase in the conductivity of X and Y rather than to the production of leaks (there is no reason to suppose that the latter process would be irreversible from the start).

It is also possible that the falling off of potential difference may be due to a loss of electromotive force (independent of or combined with a change in resistance). If the electromotive force is due to unequal mobilities of the ions or unequal partition coefficients it is quite possible that any substance which goes into solution in X and Y could induce alterations in this respect (reversible or irreversible).

In the case of Fig. 2 we may suppose that X and Y change simultaneously but this need not be a general rule. If Y should go a little before X we might get curves similar to those in Fig. 7 *b*, corresponding to the curve shown in Fig. 7 *a*, which is a tracing of the latter part of the photographic record in Fig. 8. As a matter of fact such curves are much more common than the type shown in Fig. 2.

In Fig. 8 and some of the other figures the first large movement after the application of chloroform is preceded by a small movement in the opposite direction. This will be called the counter movement. It might be explained as due to the fact that the chloroform strikes X first so that we might expect the change in X to begin a little before that of Y. Since the "counter movement" is not a constant feature and may possibly be explained in other ways it will be omitted from the theoretical diagrams in this paper.

The fact that Y is apt to change more rapidly than X might be due to differences in the layers themselves or to the solutions in contact with them. The latter seems improbable since we find the same thing when we replace 0.05 M KCl by sap or artificial sap. We may regard all of these solutions as equally "effective" in this respect (we shall use the word "effective" in this special sense throughout the subsequent discussion, the most "effective" solution being the one which most assists the action of the chloroform in changing the potential difference). On the other hand, we know from previous experiments[3] that the two layers are not identical in their properties and we may therefore assume that Y is more sensitive than X to the action of chloroform.

Let us now consider an experiment with a more dilute solution,

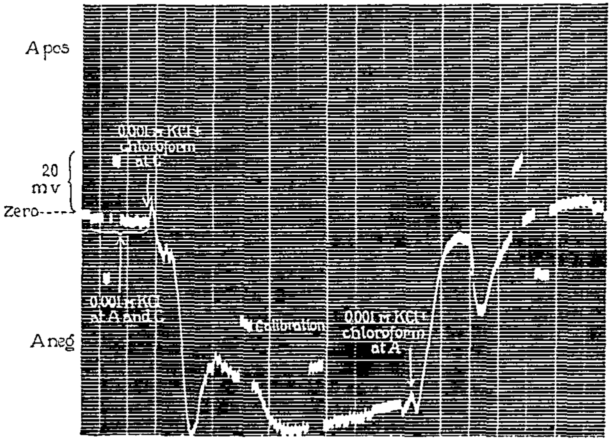

FIG. 9. Photographic record of potential differences; the experiment is arranged as in Fig. 1: at the start 0.001 M KCl is applied at A and C. After the counter movement which follows the application of 0.001 M KCl saturated with chloroform at A we see that A becomes more negative, then more positive and then approaches zero (C being dead remains constant).

The first part of the curve (resulting from the application of 0.001 M KCl saturated with chloroform to C) is similar but with signs reversed because the changes are occurring at C: after the death of C the curve reaches a fixed value which is practically all due to the living protoplasm at A. Chloroform is then applied at A giving the death curve already described.

The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

Calibration marks are shown at four places. Small irregular fluctuations are due to movements of the operator, mechanical disturbances or sound waves: they are more noticeable with dilute solutions which introduce greater resistance in series with the living cell.

namely 0.001 M KCl: the result is shown in Fig. 9. The curve shows that after C is dead A is positive:[22] hence we diagram the protoplasm

[22] This is in harmony with the results of unpublished experiments (made by us in 1922) which show that dilute solutions are positive to more concentrated solutions, as seems to be usually the case with uninjured living cells.

as in Fig. 10 with the arrow at Y[23] longer than that at X. After the counter movement the first effect of chloroform is to make A more negative;[9] hence we infer that Y changes more rapidly than X. This might be expected on the ground that it is in contact with the more concentrated solution and it appears that in general, even in the absence of chloroform, concentrated solutions render the protoplasm unstable and more easily injured. We therefore regard sap as a more effective solution than 0.001 M KCl.

In order to account for this result we may refer to the diagram in Fig. 11 *b*. We draw hypothetical curves for x and y (just as in Figs. 6 *a* and *b* and 7 *a* and *b*) in such a manner that the values of $y - x$ when plotted as in Fig. 11 *a* give us the latter part of the curve in Fig. 9 (of which the curve in Fig. 11 *a* is a tracing).

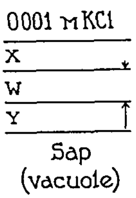

FIG. 10.

FIG. 10. Hypothetical diagram to illustrate the condition of the protoplasm in contact with 0.001 M KCl. The direction in which the positive current tends to flow is shown by the direction of the arrows, the relative magnitude of the electromotive force being indicated by their length. The potential difference across the protoplasm is said to be positive since the positive current tends to flow as in Fig. 4 *a* (since the positive arrow at Y is longer).

The variations which are observed are such as the hypothesis might lead us to expect. For example if the process in X were a little slower we should get the situation shown in Fig. 12 *b*, which would give the observed curve shown in Fig. 12 *a*. We should also expect that the y curve might sometimes drop for a time below the x curve in the latter part of its course making the potential difference temporarily negative and such cases are actually observed.

Let us now consider an intermediate concentration (0.01 M KCl): this is illustrated by Fig. 13 which shows that after C is dead A has a value[9] which approximates zero, hence in Fig. 14 we make the arrows equal. As Y is in contact with the more "effective" solution we expect it to change more rapidly and we picture the process (after the counter movement) as shown in Fig. 15 *b* from which we plot $y - x$, obtaining

[23] This presumably remains unaffected by the external solution, at least in short experiments.

the curve in Fig. 15 *a* which is a tracing of the latter part of the curve in Fig. 13 (the crossing of the curves at the right in Fig. 15 *b* is apparently a frequent phenomenon at all concentrations and results in the observed curve changing sign for a while in the latter part of its course).

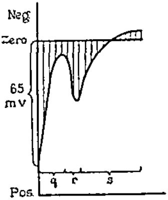

Fig. 11 *a*.

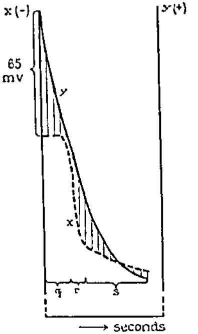

Fig. 11 *b*.

Fig. 11 a. Tracing of the latter part of the curve shown in Fig. 9 (after the counter movement). Certain ordinates are drawn for comparison with Fig. 11 b.

Fig. 11 *b*. Theoretical interpretation of Fig. 11 *a*. The ordinates as drawn represent the observed potential difference (which is equal to that of Fig. 11 *a*). Fig. 11 *a* shows only the observed potential difference but Fig. 11 *b* shows that this is equal to the difference between the value of x (with negative sign) and that of y (with positive sign). Each ordinate has the same length as the one directly above it in Fig. 11 *a*. The curve begins at the end of the counter movement when the value of A is 65 millivolts. During the period marked q the value of x remains stationary while that of y falls off as the curve in Fig. 11 *a* becomes more negative. During the period marked r the value of x falls off as the curve in Fig. 11 *a* becomes more positive. During the period marked s the curves approach each other, as the curve in Fig. 11 *a* approaches zero; after they cross the curve in Fig. 11 *a* becomes negative.

The behavior in 0.01 M KCl raises an interesting question. In the case of 0.001 M KCl it might be suggested that the layer which changes most rapidly is the one with the greatest potential difference across it. But in the case of 0.01 M KCl where the potential differences across X and Y are equal it would appear that the determining cause is the "effectiveness" of the solution rather than magnitude of the potential difference.

It will be noted that in Fig. 2 (and in Fig. 9 after the counter movement) the first result of the application of chloroform to A is a loss of potential difference. But with 0.01 M KCl even when the value of A before injury is negative we find (disregarding the counter movement) an increase on applying chloroform. For example in the latter part

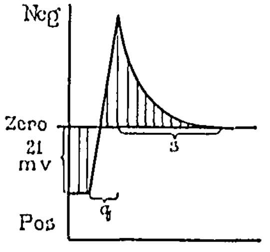

FIG. 12 a.

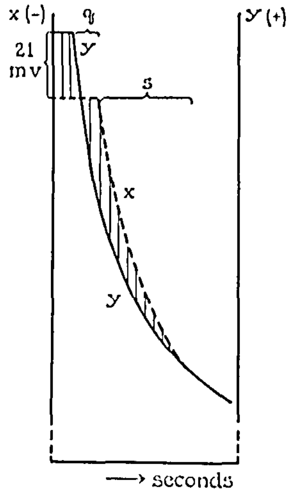

FIG. 12 b.

FIG. 12 a. Like Fig. 11 a but showing a different result.

Fig. 12 b. Theoretical interpretation of Fig. 12 a. The ordinates as drawn represent the observed potential difference which is equal to that of Fig. 12 a. Fig. 12 a shows only the observed potential difference but Fig. 12 b indicates that this is equal to the difference between the value of x (which has a negative sign) and that of y (with positive sign). Each ordinate has the same length as the one directly above it in Fig. 12 a.

During the period marked q the value of x remains constant while that of y falls as the curve in Fig. 12 a becomes more negative. During the period marked s the value of x falls off and the two curves approach each other as the curve in Fig. 12 a approaches zero.

of the curve in Fig. 16 (when C is dead but A is still normal) A is negative[24] and when chloroform is applied to A[9] it becomes more negative and then commences to fall toward zero. It would seem

[24] The fact that the potential difference across the protoplasm in contact with 0.01 M KCl is sometimes negative (Fig. 16) and sometimes zero (Fig. 13) may be due to variations in sap. It might also be due to variations in X and Y. It has been shown in a former paper[3] that these are not alike, and this accords with the diagram in Fig. 14 for if X and Y were alike we could not make the arrows in this diagram of equal length since sap is approximately equivalent to 0.05 M KCl.

that this would afford a crucial test of the ability of our hypothesis to explain all the facts. As a matter of fact it is precisely what the

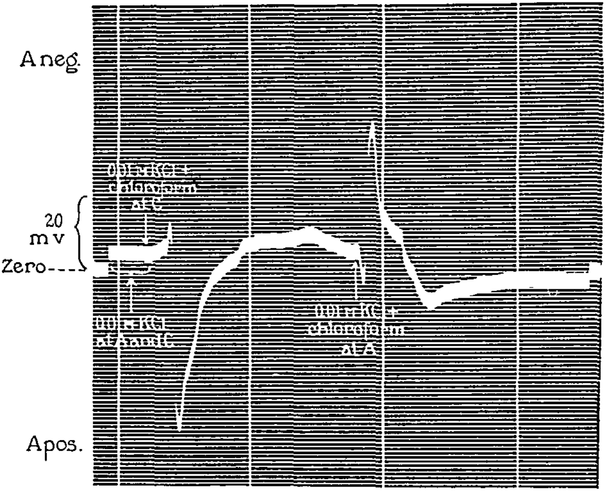

FIG. 13. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at *A* and *C*: 0.01 M KCl saturated with chloroform is applied to C and then to *A*. Just before applying chloroform to *A* it is slightly negative. When chloroform is applied we see that after the counter movement *A* becomes more negative, then more positive, and finally approaches zero (*C* being dead remains constant).

The first part of the curve (resulting from the application of chloroform to *C*) is similar but with signs reversed because the changes occur at *C*. After the death of *C* the curve reaches a fixed value which is practically all due to the living protoplasm at *A*. Chloroform is then applied to *A*, giving the death curve already described.

The vertical marks represent 20 second intervals. Selected as typical from *over 100 experiments.*

hypothesis would lead us to expect, since it demands that in all cases where V changes more rapidly the first large jump (after the counter

movement) will be in the negative direction even though (as here and in Fig. 8) the value across the protoplasm is already negative.

To interpret Fig. 16 we diagram the protoplasm as in Fig. 3 *b* and

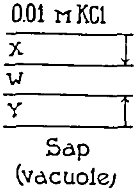

FIG. 14.

FIG. 14. Hypothetical diagram of the condition of the protoplasm in contact with 0.01 M KCl. The potential difference across the protoplasm is zero since the two arrows are equal.

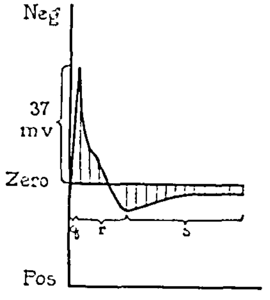

FIG. 15 *a*.

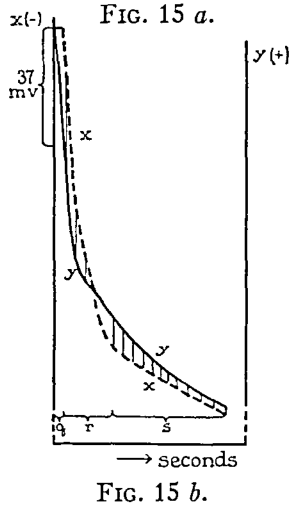

FIG. 15 *b*.

FIG. 15 *a*. Tracing of the latter part of the curve in Fig. 13 (after the counter movement) with certain ordinates drawn for comparison with Fig. 15 *b*).

FIG. 15 *b*. Theoretical interpretation of Fig. 15 *a*. The ordinates as drawn indicate that the observed potential difference is equal to the difference between *x* (with negative sign) and *y* (with positive sign) and corresponds to the observed potential difference in Fig. 15 *a* (each ordinate as drawn has the same length as the one directly above it in Fig. 15 *a*). During the period marked *q* the value of *x* remains stationary while that of *y* falls off as the curve in Fig. 15 *a* becomes more negative; during the period marked *r* the value of *x* falls off as the curve in Fig. 15 *a* becomes more positive; during the period marked *s* the two curves approach each other as the curve in Fig. 15 *a* approaches zero (this zero has no relation to the absolute zero of Fig. 15 *b*).

we picture the process (after the counter movement) as shown in Fig. 17 *a* and *b*: *Y* changes more rapidly since it is in contact with the more effective solution.

Let us now consider the effects of a more concentrated solution, namely 0.1 M KCl, as shown in Fig. 18. After *C* is dead we find that

A is negative.[23] We therefore diagram the protoplasm as in Fig. 3 *b*.[10] Since X is in contact with the more effective solution we expect it to change more rapidly and we therefore picture the process as in Fig. 19 *b* where x and y fall off in such fashion that when the values of $y - x$

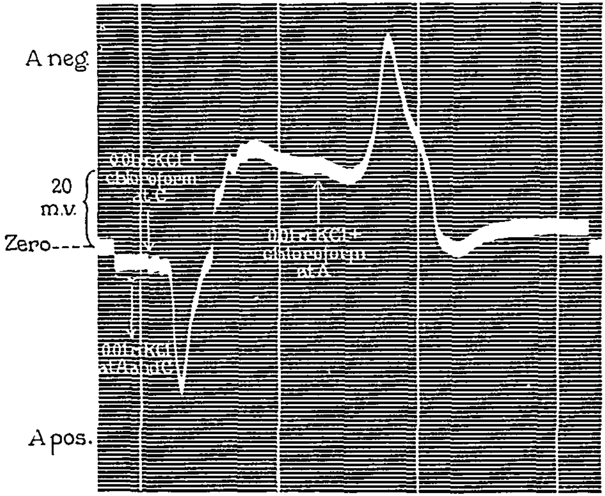

FIG. 16. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at A and C: 0.01 M KCl saturated with chloroform is applied to C and then to A. Just before applying chloroform to A its value is negative: when chloroform is applied we see that after the counter movement it becomes more negative and the potential difference then falls to zero and afterward becomes slightly negative.

The vertical lines represent 20 second intervals. Selected as typical from over 100 experiments.

are plotted we obtain the curve shown in Fig. 19 *a*, which is a tracing of the latter part of the curve[9] in Fig. 18.

Here also we expect variations similar to those found in other concentrations and this expectation is fulfilled.

The hypothesis would also lead us to predict that 0.005 M KCl would produce effects similar to those of 0.001 M KCl. The experiments show that this is so.

The success of the hypothesis in predicting the behavior of the cell under so many conditions indicates that it may be a useful guide in future investigations.

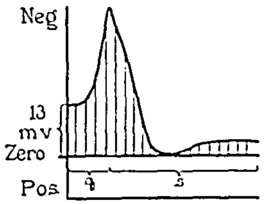

FIG. 17. *a*.

FIG. 17 *a*. Tracing of the latter part of the curve in Fig. 16 (after the counter movement) with certain ordinates drawn for comparison with Fig. 17 *b*.

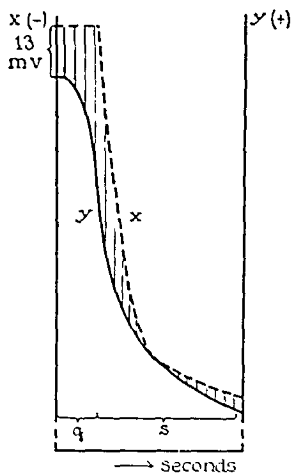

FIG. 17 *b*.

FIG. 17 *b*. Theoretical interpretation of Fig. 17 *a*. The ordinates as drawn indicate that the observed potential difference in Fig. 17 *a* is equal to the difference between *x* with negative sign and *y* with positive sign (each ordinate has the same length as the one directly above it in Fig. 17 *a*). During the period marked *q* the value of *x* remains stationary while that of *y* falls as the curve in Fig. 17 *a* becomes more negative. During the period marked *s* the two curves approach each other as the curve in Fig. 17 *a* approaches zero (this zero has no relation to the absolute zero of Fig. 17 *b*). After this the curves diverge as the curve in Fig. 17 *a* becomes more negative.

It is evident that the hypothesis would be equally satisfactory if in Figs. 3 *a* and *b*, 10 and 14 we should reverse the direction of the arrows and assume that the layer in contact with the more dilute solution is the one which changes more rapidly. It is difficult to test this experimentally with chloroform since it penetrates so quickly as to reach *Y* almost as soon as *X* but if we employ a substance which enters less rapidly, first attacking *X* and slowly making its way through *X* and *W* to attack *Y*, we may be able to decide the question.

For this purpose 0.16 M formaldehyde in 0.01 M KCl was applied at *A* (with 0.01 M KCl at *C*). The result is shown in Fig. 20; it is evident

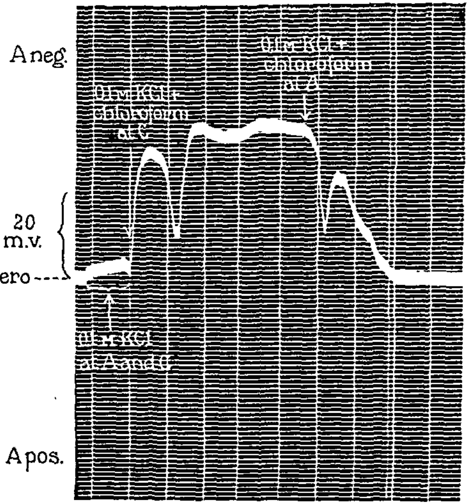

FIG. 18. Photographic record showing potential differences. The experiment starts with 0.1 M KCl at *A* and *C*: 0.1 M KCl saturated with chloroform is applied at *C* and then at *A*. Under the influence of chloroform *A* becomes more positive, then more negative, and the potential difference then falls to zero.

The first part of the curve (resulting from the application of chloroform to *C*) is similar but with signs reversed because the changes occur at *C*. After the death of *C* the curve reaches a fixed value which is practically all due to the living protoplasm at *A*. Chloroform is then applied at *A* giving the death curve already described.

The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments

that the protoplasm becomes more positive. The action is gradual, presumably because the formaldehyde penetrates slowly. This is just the opposite from the effect of chloroform, which produces a

rapid negative change as shown in Fig. 21 where 0.01 M KCl saturated with chloroform was applied at C causing its value to become more negative (it appears on the record to become more positive because the curve records the state of A with reference to C, hence the signs are reversed): 0.16 M formaldehyde was then applied at A which slowly became more positive.

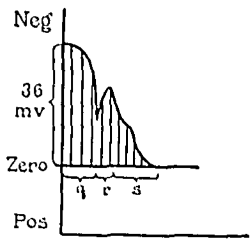

FIG. 19 *a*.

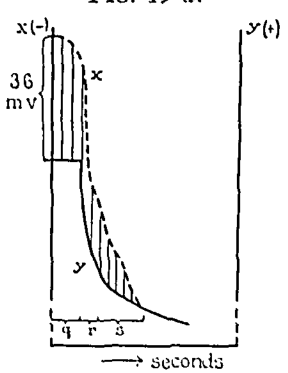

FIG. 19 *b*.

FIG. 19 *a*. Tracing of the latter part of the curve in Fig. 18 with certain ordinates drawn for comparison with Fig. 19 *b*.

FIG. 19 *b*. Theoretical interpretation of Fig. 19 *a*. The ordinates as drawn indicate that the observed potential differences in Fig. 19 *a* are equal to the difference between x with negative sign and y with positive sign (each ordinate has the same length as the one directly above it in Fig. 19 *a*). During the period marked q the value of y remains constant while that of x falls off as the curve in Fig. 19 *a* becomes more positive; during the period marked r the value of y falls off as the curve in Fig. 19 *a* becomes more negative; during the period marked s the two curves approach each other as the curve in Fig. 19 *a* approaches zero (this zero has no relation to the zero of Fig. 19 *b*).

These results indicate that the X arrow has the direction shown in the figures. This does not seem to be in harmony with the widely accepted view that the inside of the plasma membrane is negative to the outside[25] for in that case the arrow at X would have to be reversed. The idea that the inside of the plasma membrane is negative was adopted to account for the negative current of injury but it seems probable that in *Nitella* at least the negative current of injury described in the literature can be accounted for in a different way. The

[25] If, as we assume, the inside of X is positive we should find on inserting a capillary through X so as to lead off from the inside of X to the outside that the positive current would flow from the inside through the capillary and the electrometer to the outer surface of X.

negative currents of injury described in this paper may be too brief to correspond to the traditional negative current of injury referred to in the literature and it is questionable to what extent they could be observed in cells of ordinary size with the methods usually employed.

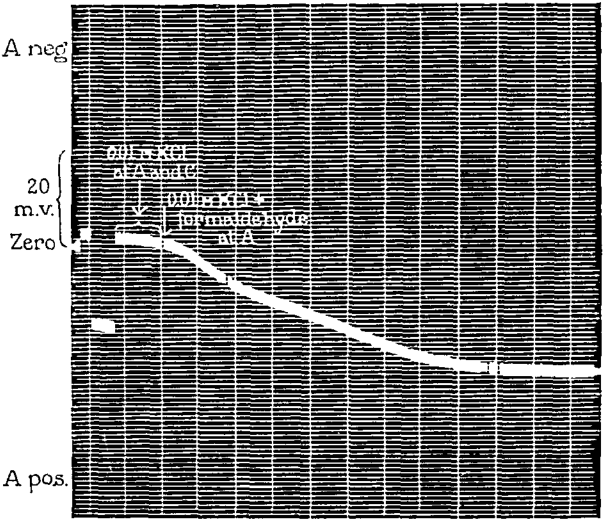

FIG. 20. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at *A* and *C*: then 0.16 M formaldehyde in 0.01 M KCl is applied at *A* (with 0.01 M KCl at *C*). Formaldehyde makes *A* more positive but the action is gradual.

The vertical lines represent 5 second intervals. Selected as typical from 30 experiments.

In order to obtain results comparable with those described in the literature it would seem to be necessary to make experiments with portions of a plant of *Nitella* consisting of two or more cells. If we do this we find, on cutting or crushing a cell at one end, that sap, escaping at the opposite end and coming in contact with a neighboring intact cell, causes the injured cell to appear more negative and this

condition may persist for a long time. This will be fully discussed in a subsequent paper.

It will be remarked that in the previous discussion no account is taken of any possible potential differences in *W*: this does not imply that none exist but only that they are regarded as negligible for our present purpose. It would be possible to set up another hypothesis which would attribute more importance to *W*. For example, in the

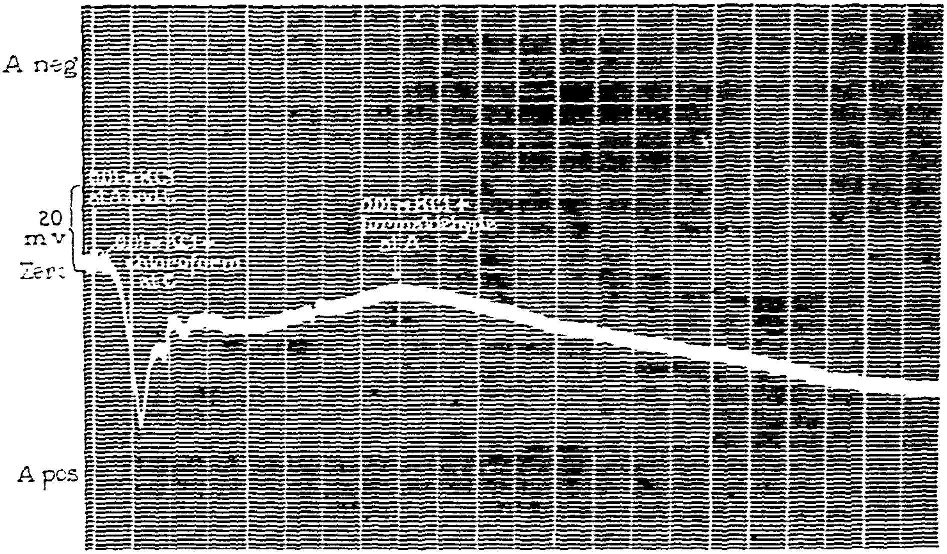

FIG. 21. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at *A* and *C*. The application of 0.01 M KCl saturated with chloroform to *C* causes it to become more negative (the curve becomes positive because the signs are reversed owing to the fact that the change occurs at *C* but the curve shows the state of *A* rather than that of *C*). During this time *A* is in contact with 0.01 M KCl. Formaldehyde (0.16 M in 0.01 M KCl) applied to *A* causes it to become more positive. Compare with Fig. 20.

The vertical lines represent 5 second intervals. Selected as typical from 10 experiments.

case of 0.001 M KCl we might suppose that both *X* and *Y* are simultaneously destroyed leaving *W* exposed to 0.001 M KCl on the outside and to sap on the inside: if *W* reacts in the opposite way[26] from

[26] Beutner (Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben. Stuttgart, 1920), experimenting on organic substances immiscible with water, found that basic substances give an effect opposite to that of acid substances with such salts as KCl.

X and Y (*e.g.*, if, in contact with W 0.001 M KCl is negative to sap and to 0.05 M KCl) the value across the protoplasm would change from positive (as in Fig. 4 *a*) to negative (as in Fig. 4 *b*).

But this hypothesis would not account for such cases as those shown in Figs. 9 and 18 where the curve moves first in one direction and then in the other, nor for Fig. 16 where the value of A is negative and becomes more so, nor would it explain why a spot treated with sap + chloroform commonly behaves as shown in Fig. 8, since in that case W would have sap on both sides and unless we assume that W is composed of two unlike layers we should expect that when chloroform is applied the potential difference would at once fall to zero.

Another possible explanation is that the application of chloroform alters the protoplasm in such fashion as to let sap pass out (at least momentarily) and come in contact with the outside of X without altering its power to give a potential difference. If we had 0.001 M KCl at the outside at the start it would be changed to the equivalent of something between 0.001 M and 0.05 M KCl by the coming out of sap. This would lessen the potential difference and might even make it negative. On the other hand 0.1 M KCl which gives a negative value across the protoplasm would be diluted by the coming out of sap and would become less negative but it could never become temporarily positive, as often happens in cutting, because if the 0.1 M KCl were completely changed to sap the value would still be negative (as shown in a previous paper[3]). In addition this hypothesis fails to explain why a spot treated with sap saturated with chloroform commonly behaves as shown in Fig. 8.

After the completion of this paper we received through the kindness of Professor Jost a reprint of his paper[27] which deals with *Chara* and *Valonia*. He makes no mention of experiments with chloroform but his results with ether, ethyl alcohol, amyl alcohol, cutting and crushing agree (except in minor details) with ours which have been repeated many times during the last 5 years. We think it desirable, however, to make it clear that we interpret the lasting negative current of

[27] Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch., Abteilung B*, 1927, Abhandlung 13, Nov.

injury, observed when we lead off with dilute solutions from a cut or crushed cell, as due to an effect upon the neighboring intact cell (from which we are in effect leading off at one point with a dilute solution and at another with sap escaping from the injured cell, the circuit passing through the injured cell). If the solution applied to the uninjured cell is as "effective" as sap the current of injury will be of relatively brief duration. But if the solution applied to the uninjured cell is more "effective" than sap the current of injury may last for a long time and will be positive.

In view of the fact that the current of injury is sometimes explained by differences in acidity it may be well to state that varying the pH value from 5 to 8 produces relatively little effect as long as the concentration of cations other than H^+ is not changed.

SUMMARY.

Experiments on single multinucleate cells of *Nitella* show that the current of injury may be made positive or negative. For example, with chloroform in 0.1 M KCl the current of injury is positive but with 0.001 M KCl it is negative.

The changes which occur during the process of death receive a simple explanation upon the basis of the theory of protoplasmic layers. It seems possible that each layer has a death curve of simple and regular form, the more rapid alteration of the outer layer producing a positive current of injury and the more rapid alteration of the inner giving rise to a negative current of injury.

OSMOSIS OF LIQUIDS.

General Considerations.

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The present paper is a continuation of the series entitled "Equilibria in systems, in which the phases are separated by a semipermeable membrane," which have appeared in the proceedings of the Koninklijke Akademie van Wetenschappen (Amsterdam, Holland). In this paper I shall discuss in a simple form some of these results, *viz.* the osmosis of ternary liquids. The reader can find the thermodynamic deductions in the original communication.[1]

I.

Graphical Representation.

We shall begin by discussing briefly in what way ternary liquids are represented. We take a liquid, consisting of the substances X, Y, and W, for example, of sugar, urea, and water, or of salt, soda, and water, etc. If 1 gm. of this liquid contains x gm. of the substance X and y gm. of the substance Y, then it will consequently contain $1 - x - y$ gm. of the substance W, and we represent its composition by

$$x \text{ gm. } X + y \text{ gm. } Y + (1 - x - y) \text{ gm. } W$$

Instead of 1 gm. of this liquid we may also take a quantity consisting of 1 gm. mol in all; if in it x gm. mol X and y gm. mol Y are present and consequently $1 - x - y$ gm. mol W, then we represent it by

$$x \text{ gm. mol } X + y \text{ gm. mol } Y + (1 - x - y) \text{ gm. mol } W$$

[1] Schreinemakers, F. A. H., *Proc. K. Akad. Wetensch. Amsterdam*, Part 27, 701, and the 22 following communications.

In general, therefore, we may represent the composition of a liquid by x quantities of $X + y$ quantities of $Y + (1 - x - y)$ quantities of W in which "quantities" may denote gm. as well as gm. mol.

We represent the composition of this liquid by a point of a rectangular triangle $W\,X\,Y$ (Fig. 1); we take $W\,X = W\,Y = 1$.

As the liquid contains x quantities of X and y quantities of Y, we take $W\,b = x$ and $W\,a = y$; then point q represents the X and Y quantities of the liquid, we now have $qa = x$ and $qb = y$. From the figure follows $qb = Wa$ and $qa + qc = ac = aY$; as $Wa + ay = 1$, we find:

$$qa + qb + qc = 1$$

or

$$x + y + qc = 1$$

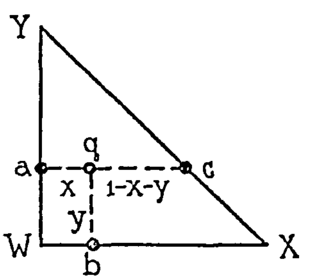

FIG. 1.

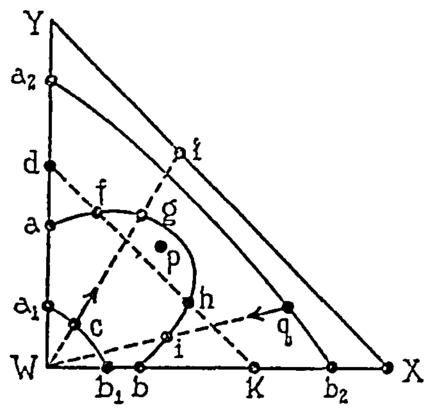

FIG. 2.

Consequently $qc = 1 - x - y$. The line qc represents, therefore, the W amount of the liquid. Consequently the point q represents a liquid which contains $qa = x$ quantities of X, $qb = y$ quantities of Y, and $qc = (1 - x - y)$ quantities of W; therefore it not only indicates the X and Y quantities but also the W quantity of the liquid. If point q is situated on side $W\,X$ then $y = 0$; the liquid then contains only W and X. If point q is situated on side $W\,Y$ then $x = 0$; the liquid then contains only W and Y. If q is situated on side XY then $(1 - x - y) = 0$; the liquid then contains only X and Y.

If q coincides with the angular point W, then $x = 0$ and $y = 0$

and $(1 - x - y) = 1$; the liquid then consists of W (water) only. In a corresponding way we see that point X represents the pure substance X and point Y the pure substance Y.

Consequently we find the angular points of the triangle represent the three pure substances, W, X, and Y: the sides represent binary liquids, *viz.* liquids which contain either $W + X$ only or $W + Y$ only or $X + Y$ only; the points within the triangle represent the ternary liquids which contain $X + Y + W$.

In Fig. 1 we draw an imaginary line through point q, parallel to XY; then in all points of this line $1 - x - y$ has the same value; this means that all liquids of this line have the same W amount. This W amount grows larger the nearer this line runs to point W.

If in Fig. 2 we imagine, for example, dK parallel to XY then the liquids d, f, h, and K must have the same W amount; this will be larger than that of the liquids g, p, q, etc., which are situated on one side of this line, but smaller than that of the liquids a, b, c, i, etc., which are situated on the other side.

We take two liquids, L_1 and L_1', with the respective compositions:

$$x_1 \text{ quant. of } X + y_1 \text{ quant. of } Y + (1 - x_1 - y_1) \text{ quant. of } W$$
$$x_1' \text{ quant. of } X + y_1' \text{ quant. of } Y + (1 - x_1' - y_1') \text{ quant. of } W$$

If we mix n_1 quantities of L_1 with n_1' quantities of L_1' we get $n_1 +$ n_1' quantities of a new liquid L, the composition of which we represent by

$$x \text{ quant. of } X + y \text{ quant. of } Y + (1 - x - y) \text{ quant. of } W$$

As those $n_1 + n_1'$ quantities of L contain in all $n_1 x_1 + n_1' x_1'$ quantities of X and $n_1 y_1 + n_1' y_1'$ quantities of Y, it follows that:

$$x = \frac{n_1 x_1 + n_1' x_1'}{n_1 + n_1'} \qquad y = \frac{n_1 y_1 + n_1' y_1'}{n_1 + n_1'} \tag{1}$$

so that the composition of the new liquid L is known. We represent those liquids L_1 and L_1' in Fig. 3 in which the sides $W X$ and $W Y$ have only been drawn partially by the points 1 and 1′, and liquid L by point e. With the aid of (1) we now can show: point e is situated on line 1-1′ and divides it into the parts 1-e and 1′-e; for the length of those parts is valid:

$$1\text{-}e : 1'\text{-}e = n_1' : n_1$$

Of the many things which may be deduced from this we shall only discuss those which will serve our purpose later on; among other things we find: If we continuously add to a certain quantity of liquid L_1' liquid L_1, then L_1' proceeds along the line 1-1′ starting from point 1′ towards point 1. If to liquid q of Fig. 2 water is added, it will, therefore, proceed along line qW in the direction of the arrow; consequently it is continually coming nearer to point W. If we withdraw water from a liquid, then this will move in the opposite direction; if, for example, we withdraw water from liquid c (Fig. 2) then this will proceed along the line $c\ l$ in the direction indicated by the arrow.

We shall now call $n_1 \times L_1 + n_1' \times L_1'$ a complex of n_1 quantities of L_1 and n_1' quantities of L_1'. This is represented in Fig. 3 by point e. It is of no account here whether the liquids of this complex mix with one another totally or partially or not at all or whatever might result from them. If, for example, n_2 quantities of L_2 and n_2' quantities of L_2' (in Fig. 3, represented by the points 2 and 2′) should be formed, then line 2-2′ must, therefore, also go through point e and we have:

$$2\text{-}e : 2'\text{-}e = n_2' : n_2 \tag{3}$$

The same is valid when from this complex the liquids 3 and 3′ or 4 and 4′ or others are formed.

II.

Osmosis of One Substance.

Positive and Negative, Normal and Anomalous Osmosis.

We imagine the liquids L and L_0 separated from one another by a membrane; we represent this by:

$$L \mid L_0 \tag{4}$$

We call this an "osmotic system" and we shall say that the liquids are in "osmotic contact." We now assume that temperature and pressure are equal on both sides of the membrane and that this is permeable only for W (water) and consequently not for X and Y.

We now can distinguish two cases, *viz.* either W diffuses in the one

or the other direction through the membrane or nothing happens. In the latter case the system is in "osmotic equilibrium;" then we may say also that the liquids are "isotonic" or that they have the same O.W.A. (osmotic water attraction).

If we imagine L_g to be represented in Fig. 2 by point g then we may put the question: what liquids have the same O.W.A. as liquid g? We may deduce an infinite number of liquids satisfying this condition; they are all situated on a curve, going through point g, and indicated in Fig. 2 by $a\ b$. All liquids of this curve, therefore, have the same O.W.A. and are consequently isotonic; for this reason we call this curve an "isotonic curve." If we take the liquids f and g, for example, we consequently have the system:

$$L_f \mid L_g \tag{5}$$

in which no water diffuses through the membrane and which, therefore, is in osmotic equilibrium.

If we take, for example, the osmotic system

$$L_a \mid L_b \tag{6}$$

we have on the left side a liquid consisting of $W + Y$ only and on the right side a liquid consisting only of $W + X$; both, however, have the same O.W.A. and are, therefore, isotonic. Each of these liquids a and b is also isotonic with all other liquids of curve $a\ b$, consequently with liquids containing the three substances X, Y, and W.

It is evident now that in Fig. 2 an infinite number of isotonic curves may be drawn; we find in this figure the curves $a_1\ b_1$, $a\ b$, and $a_2\ b_2$. All liquids of $a_1\ b_1$ have, therefore, the same O.W.A., also all liquids of $a\ b$ and also those of $a_2\ b_2$. The O.W.A. of the liquids of curve $a_1\ b_1$ is smaller, however, than that of curve $a\ b$ and their O.W.A. is smaller again than that of the liquids of curve $a_2\ b_2$. We now can show that the O.W.A. of the liquids of an isotonic curve grows, the farther this curve is away from point W.

We may among others deduce the following properties of those curves.

1. Two isotonic curves never can intersect or touch one another.

2. Every straight line going through point W intersects a curve in one point only.

3. The O.W.A. of the liquids of an isotonic curve is greater, the farther this curve lies from point W.

4. The isotonic curves are straight lines in the vicinity of point W; at a greater distance they are curved and may take various shapes. We shall refer to this later on.

Now we may put the question: What will happen if we bring two liquids in osmotic contact with one another?

We can show now that: if both liquids have the same O.W.A. nothing happens; if both liquids have a different O.W.A. water diffuses from the liquid with the smaller O.W.A. towards that with the larger. This osmosis continues till both liquids get the same O.W.A.

An isotonic curve, for example a b, divides the triangle into two parts; from the above mentioned property, (3) follows: all liquids of field W a b have a smaller, and all liquids of field a b X Y have a larger O.W.A. than those of curve a b.

If, therefore, we take the osmotic system:

$$L_c \mid L_q \rightarrow \tag{7}$$

(Fig. 2) then the right side liquid has a larger O.W.A. than the left side liquid; consequently the water diffuses through the membrane towards the right, as has been represented in (7) by an arrow.

As liquid q absorbs water, it, therefore, proceeds along line q W in the direction of the arrow in Fig. 2; as liquid c loses water, it proceeds along line c l in the direction of the arrow in point c. Consequently the O.W.A. of liquid q decreases continuously (*viz.* it comes continuously on isotonic curves, which are situated closer to point W); the O.W.A. of liquid c, however, increases continuously; the diffusion of the water will continue till both liquids get the same O.W.A.; this is the case when they come on the same isotonic curve. If we assume that a b is this curve, then at the end of the osmosis liquid c must come in g and liquid q in i. Consequently system (7) passes into the osmotic equilibrium:

$$L_g \mid L_i \tag{8}$$

(Fig. 2) in which no water diffuses any more through the membrane. Consequently during the osmosis liquid c has proceeded along the straight line c g and liquid q along the line q i; we call those lines the

"osmosis path" of the liquids or of the system. Later on we shall see that those paths are not straight lines, but curves, when more than one substance diffuses through the membrane.

From our considerations it follows that liquids with a similar W amount need not yet be isotonic. This is clear; as we can show that the O.W.A. also depends on the nature of the other substances, present in the liquid.

Let us take Fig. 2, for example, in which line $d\,K$ is parallel to side $X\,Y$, so that all liquids of this line have the same W amount. On this line we take a liquid, h. Of all other liquids of this line $d\,K$ there is, therefore, only one, *viz.* f, which has the same O.W.A. as liquid h. All liquids which are situated between f and h have a smaller O.W.A. than liquid h and all which are situated either between d and f or between K and h have a larger O.W.A. than liquid h.

Consequently if we take a liquid u between f and h we have a system:

$$L_u \mid L_h \longrightarrow \tag{9}$$

in which the water diffuses towards the right; if we take u between d and f or between K and h, we have a system:

$$L_u \mid L_h \longleftarrow \tag{10}$$

in which the water goes through the membrane towards the left. In both systems (9) and (10) the liquids on the left and the right side of the membrane have the same W amount; yet in (9) water diffuses towards the right and in (10) towards the left. Consequently it is apparent from this that if two liquids with a similar W amount are in osmotic contact with one another, in most cases diffusion of water will occur after all.

It is also possible that the water diffuses from a liquid with smaller W amount towards a liquid with larger W amount. Let us, for example, take the system:

$$L_p \mid L_h \longrightarrow * \tag{11}$$

(Fig. 2) in which the left side liquid p has a smaller W amount than the right side liquid h. If, however, we imagine an isotonic curve through p, then we see that this is situated closer to point W than

curve *a b* on which point *h* is found. Consequently liquid *h* has a larger O.W.A. than liquid *p*; therefore, in (11) the water diffuses towards the right, that is to say from the liquid with the smaller to the liquid with the larger *W* amount.

In order to distinguish easily the many cases which may occur with the osmosis of one, but especially of more substances, we shall use some signs. We shall indicate by the sign $<$ that the left side liquid has a smaller amount of *W* than the right side liquid; consequently the sign $>$ indicates that the left side liquid has a larger amount of *W*. We now distinguish four cases. The first two we represent by

$$(a) \qquad \xleftarrow{<} \qquad\qquad \xrightarrow{<}{}^{*} \qquad (12)$$

In both cases the liquid on the left side of the membrane has, therefore, a smaller *W* amount than the liquid on the right side; the arrows indicate the direction, in which the water diffuses.

In the first case, therefore, the water diffuses from a liquid with larger to a liquid with smaller *W* amount; we call this a "positive" osmosis.

In the second case the water diffuses in the opposite direction, *viz.* from the liquid with smaller to that with larger *W* amount; we call this a "negative" osmosis. In order to make this strike the eye at once, we shall henceforth put an asterisk with arrows, which indicates a negative osmosis.

The two other cases are:

$$(b) \qquad \xleftarrow{>}{}^{*} \qquad\qquad \xrightarrow{>} \qquad (13)$$

Intrinsically they absolutely resemble the first two; the only difference being that now the left side liquid has a greater *W* amount than the right side liquid. Indeed, in the first of these cases the osmosis is negative, in the second positive. In (11) we have already discussed an example of negative osmosis; this is indicated by the sign * with the arrow.

As, during the osmosis, the liquids are changing their concentrations all the time, we shall also represent those changes by schemes. Later on, especially when more substances diffuse, we shall see that this will give us a clear survey. We assume that the liquid on the left

side of the membrane has a smaller W amount than the liquid on the right side. We now distinguish four cases:

1. During the osmosis the amount of W increases on the left side of the membrane and decreases on the right side. We represent this by

$$\uparrow < \downarrow \tag{14}$$

The left side arrow which is pointed upward indicates that the amount of W of the left side liquid increases; the right side arrow which is pointed downward indicates that the amount of W of the right side liquid decreases.

2. The W amount of both liquids increases; we represent it by

$$\uparrow < \uparrow * \tag{15}$$

To the meaning of the sign * we shall refer later on.

3. The W amount of both liquids decreases,

$$* \downarrow < \downarrow \tag{16}$$

4. The W amount of the left side liquid decreases and that of the right side liquid increases

$$* \downarrow < \uparrow * \tag{17}$$

In subsequent communications we shall discuss experimental examples of such cases.

Now we shall say:

A. The W amount of a liquid changes "normally" (1) if it increases when the liquid on the other side of the membrane has a larger amount of W; (2) if it decreases when the liquid on the other side of the membrane has a smaller amount of W.

B. The W amount of a liquid changes "anomalously" (1) if it increases although the liquid on the other side of the membrane has a smaller amount of W; (2) if it decreases, although the liquid on the other side of the membrane has a larger amount of W.

All we have discussed above as regards the W amount of a liquid, is valid also for its X and Y amount. We see that in scheme (14) both liquids change normally; now we shall say that the system changes normally-normally.

In scheme (15) the left side liquid changes normally, the right side liquid, however, changes anomalously. In order to make this clear at once, we put an asterisk near the arrow, which indicates an anomalous change of a concentration. We say that this system changes normally-anomalously.

In scheme (16) we shall say that the system changes anomalously-normally.

In scheme (17) it changes anomalously-anomalously.

Of course, the words normal and anomalous should not lead us to look upon the one phenomenon as more normal than the other.

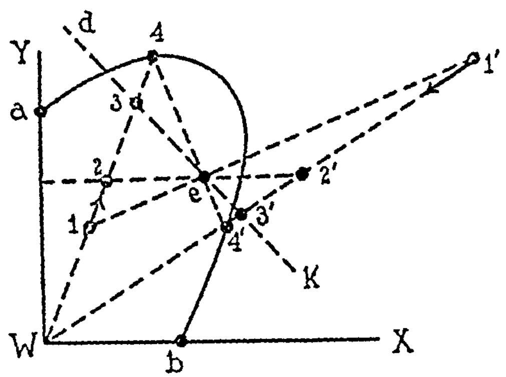

FIG. 3.

We imagine the liquids L_1 and L_1' of the osmotic system

$$L_1 \mid L_1' \tag{18}$$

(Fig. 3) to be represented by the points 1 and 1'. If curve a 4-4' b is an isotonic curve, then it follows that the right side liquid L_1' will have a larger O.W.A. than the left side liquid L_1, consequently the water diffuses toward the right. The left side liquid loses water; consequently starting from point 1 it goes in the direction of the arrow; the right side liquid, which absorbs water, must go in the direction of the arrow starting from point 1'. When both liquids on curve a b get the same O.W.A. then the left side liquid L_1 will proceed along the line 1-4 and the right side liquid along the line 1'-4'.

If the left side liquid is at a certain moment somewhere in a point u of the line 1-4, then the right side liquid is somewhere in a point u' of the line 1'-4'. Those points u and u' are not situated arbi-

trarily, however, for if we have n_1 quantities of L_1 and n_1' quantities of L_1', then, as we have seen before, the complex $n_1 \times L_1 + n_1' \times L_1'$ is represented by a definite point c of the line 1-1'; as the liquids u and u' arise from this complex, the line $u\ u'$ must always go through point c. If liquid u is found in 2, then u' must be in point 2'; when liquid u is found in 3, then u' must be in point 3', etc.

During this osmosis the X, Y, and W amounts of the liquid change; we shall begin by considering the X amount.

From Fig. 3 it appears that the X amount of the left side liquid increases from point 1 until 4; the X amount of the right side liquid decreases from point 1' until 4'. We also see that the X amount of the left side liquid during all the osmosis is smaller than that of the right side liquid. Consequently we can represent this by

$$\uparrow < \downarrow \qquad (19)$$

We can say, therefore, that the X amount of the liquids changes normally-normally.

From Fig. 3 it appears also that the Y amount of the left side liquid increases from point 1 until point 4 and that the Y amount of the right side liquid decreases from point 1' until 4'. We now imagine the line 2-2' to be horizontal; we may now distinguish two cases.

If the left side liquid is found on part 1-2 and the right side liquid, therefore, on 1'-2' then the left side liquid must have a smaller Y amount than the right side liquid; consequently we get the scheme:

$$1\text{-}2 \quad \uparrow < \downarrow \qquad (20)$$

in which is indicated also that this is valid for part 1-2 of the path.

If, however, the left side liquid is on the part 2-4 and hence the right side liquid on 2'-4' then the left side liquid has a greater Y amount than the right side liquid, we then have the scheme:

$$2\text{-}4 \quad {}^*\!\uparrow > \downarrow^* \qquad (21)$$

Although the Y amount on the left side of the membrane is larger than on the right side, yet it increases on the left side and it decreases on the right side; hence the Y amount changes on part 2-4 anomalously-anomalously.

It appears from Fig. 3 that the W amount of the left side liquid

decreases from point 1 until point 4; the W amount of the right side liquid increases from point 1′ until point 4′. We now imagine the line $d\ K$ to be parallel to the side $X\ Y$, which has not been drawn; we can distinguish two cases.

If the left side liquid is on part 1-3 and the right side liquid, therefore, on 1′-3′ then the left side liquid must have a larger W amount than the right side liquid, so that we have the scheme:

$$1\text{-}3 \quad \underrightarrow{\downarrow > \uparrow} \tag{22}$$

in which is indicated also the direction in which the water diffuses through the membrane.

If, however, the left side liquid is on part 3-4 then it has a smaller W amount than the right side liquid, which is then on part 3′-4′; we then get the scheme:

$$3\text{-}4 \quad {}^*\underrightarrow{\downarrow < \uparrow}{}^*_* \tag{23}$$

Although the W amount is smaller on the left side of the membrane than on the right side, the water diffuses toward the right, notwithstanding; consequently we have a negative W osmosis; also both liquids change their W amount anomalously; consequently we might say that the system changes its W amount anomalously-anomalously-negatively.

It is not necessary to use the horizontal arrows in the schemes (22) and (23), for it follows already from the vertical ones that the W amount decreases on the left side and increases on the right side, so that we may conclude that the water diffuses toward the right. If, however, more than one substance passes through the membrane, then, as we shall see later on, we are not allowed to draw a conclusion from the vertical arrows as to the direction in which the substance passes through the membrane; this conclusion might be totally mistaken and make it absolutely necessary to indicate this direction by a horizontal arrow.

We may summarize what has been deduced above in a single scheme (24)

Fig. 3	X	Y	W	
1-2	$\uparrow < \downarrow$	$\uparrow < \downarrow$	$\underrightarrow{\downarrow > \uparrow}$	
2-3	"	$^*\uparrow > \downarrow^*$	"	(24)
3-4	"	"	${}^*\underrightarrow{\downarrow < \uparrow}{}^*_*$	

In this scheme we find the three parts into which we must divide the line 1-4; under each of the substances X, Y, and W has been indicated what we have deduced above with regard to these substances; in it, therefore, we find everything that may be deduced from Fig. 3.

We see, for example, that on part 1-2 all concentrations change normally; on part 2-3 and 3-4, however, the Y amount and on part 3-4 the W amount change anomalously. On the parts 1-2 and 2-3 we have a positive osmosis, on part 3-4, however, a negative osmosis. In systems, in which more substances diffuse through the membrane, we have already found experimentally several examples of anomalous change of concentration and of negative osmosis.

ON THE MECHANISM OF TONIC IMMOBILITY IN VERTEBRATES.

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I.

Tonic immobility (Crozier, 1923), often more popularly known as "playing possum," "animal hypnosis," or even "feigning death," is exhibited by a great variety of animals. Rabaud (1919) discusses at length the condition as seen in arthropods. He points out that most arthropods have regions of the body which seem to be the loci of tactually excitable reflexes causing the animal to pass into a state of immobility characterized by heightened tonicity of the skeletal musculature. The durations of the periods of immobility vary considerably from species to species, and to a lesser degree for individuals within the species. The antennæ, thorax, and bases of the wings are in general regions which when stimulated tend to produce immobilization; while other parts of the body, particularly the feet, seem to be the seat of antagonistic reflexes causing recovery of activity. The state is conspicuous by the fact of its apparent all-or-none nature and because of the characteristic positions assumed by the animals.

Verworn in 1898 discussed tonic immobility as seen among lower vertebrates, and Mangold (1920) has described the condition in higher vertebrates. With birds and mammals the state is most readily produced by suddenly turning the animal dorsum downward. Guinea pigs are more readily immobilized by this method than most mammals, although the condition has been produced in young dogs, cats, apes, and sheep. Among birds the state is well demonstrated

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in hens and pigeons. As early as 1562 the Abbé Kircher records the phenomenon in a hen (*cf.* Piéron, 1913; Mangold, 1920).[1]

Foxes and opossums are said to "feign death" sometimes when pursued by enemies and to remain quite immobile even if mauled about. In such cases it has been urged that the phenomenon is a death feint of definite survival value, and as a result it has been spoken of as an "instinct of death-feigning."

In all cases from cœlenterates to mammals the condition is marked by a high degree of plastic tonus of skeletal musculature and a pronounced lack of reactivity to environmental disturbances. Verworn (1898) speaks of it as animal hypnosis, because of the similarity of the plastic tonus to that shown in the cataleptic trance of the human hypnotic subject. The term, however, is not a very happy one in this connection because of its psychological implications.

Work by Rabaud (1919) and also by Holmes (1906) has tended to show that in arthropods the condition is not dependent on any one neurone center. On severally destroying various ganglia the state has been found to continue. *Ranatra* even when cut in two continues the "feint" for its customary period (Holmes, 1906).

Crozier (1923) gave a summary of work with the isopod, *Cylisticus convexus*. By correlation with the Arrhenius equation[2]

$$\frac{k_2}{k_1} = e^{\frac{\mu}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)},$$

[1] Tonic immobility or a state akin to it has been described in children by Piéron (1913). I have recently been able to produce the condition in adult human beings. The technique was brought to my attention by a student in physiology, Mr. W. I. Gregg, who after hearing a lecture on tonic immobility suggested that a state produced by the following form of manhandling which he had seen exhibited as a sort of trick might be essentially the same thing. If one bends forward from the waist through an angle of 90°, places the hands on the abdomen, and after taking a deep breath is violently thrown backwards through 180° by a man on either side, the skeletal muscles contract vigorously and a state of pronounced immobility lasting for some seconds may result. The condition is striking and of especial interest since this type of manipulation (sudden turning into a dorsal position) is the most common one used for producing tonic immobility in vertebrates.

[2] *Cf.* Taylor, 1924; Crozier, 1924, *a*, *b*.

he examined the effect of temperature on successively induced periods of tonic immobility and concluded the effects might be accounted for by assuming the durations proportional to the amount of an inhibitory substance which was the mid-product in a catenary series. Two values of μ, the temperature characteristic, were obtained (24,000 and 9,200) from lines of different slope meeting at a critical temperature of 16°.

A similar investigation was undertaken with vertebrates. The mechanism of tonic immobility in vertebrates is of interest since there is invoked a general hypertonicity of the skeletal muscles apparently associated with widespread inhibition of impulses from the so called "higher centers." Tonic immobility is useful as a possible means of studying tonus and selective inhibition, and is actually a special case of the problem of alternate periods of movement and quiescence of an organism.

II.

Birds and mammals are out of the question for such experiments because of the impossibility of changing the controlled temperature. The lizard *Anolis carolinensis* is readily immobilized by turning it into the dorsal position and applying pressure lightly with the finger on the thorax. The onset of immobility is usually marked by a period of deep gasping respirations, followed by a gradual decrease in amplitude and frequency of respiratory movement until breathing is hardly perceptible. The immobile state is characterized by a heightened tone of the leg muscles and by a certain degree of non-reactivity to the environment although the eyes frequently remain open and alert. *Anolis* may remain immobile for periods ranging from a few seconds to several hours, depending upon conditions presently to be discussed.

The recovery of activity is well marked and even dramatic. It is generally heralded by the progression of a wave of activity starting at the tip of the tail and passing forward to the lumbar region, when the animal springs violently into activity; if lying immobile on the back it leaps to its feet and endeavors to escape.

Owing to a comparative responsiveness to environmental disturbances during the immobile state great difficulty was at first found in working with *Anolis*. It was desired to begin with the application of the temperature method of analysis.

Attempts were made in preliminary experiments to measure with a stop-watch the successive intervals between induction of immobility and spontaneous recovery. It soon became apparent, however, that this was a difficult task since the mere movement of objects in the environment was often sufficient to bring about a premature recovery and so to interfere with possible cyclical phenomena. Moreover, it was evident from the start that a large amount of data would be required for significance in statistical treatment. This necessitated watching the animal for long periods of time in order to ascertain the durations of the periods.

Because of the instability of the immobile state of *Anolis* another lizard was obtained, the horned "toad" *Phrynosoma cornutum.* This animal is readily put in a stable immobilized condition by a procedure similar to that used for *Anolis. Phrynosoma* seems to be quite indifferent to most environmental disturbances, so that once immobilized it continues the "feint" even though moderately handled.

Automatic immobilizing and recording had to be devised, since excessive durations of 5 to 10 hours were sometimes encountered. Ideally a method was desired whereby the animal could be automatically reimmobilized as soon as it recovered. This should be accomplished by a procedure in which light and temperature conditions could be kept constant and in which the excitement incident to handling and immobilizing the animal should be minimized.

Control of temperatures above that of the room was effected by the use of a Freas electrically regulated air incubator. For low temperatures a thermostat (Crozier and Stier, 1927) was used consisting, in the main, of a water bath with a stirrer in which is immersed a cooling coil connected to a motor-driven SO_2 condenser pump and external air-cooled coil. The motor of the pump is started and stopped by the action of a relay system which is in turn controlled by a mercury thermoregulator in the bath.

Preliminary experiments were made in which the animal was fastened in a holder, ventral side up, within the thermostat. One of its legs was connected by levers to a switch operating a signal magnet against a smoked drum. An automatic, electrically driven mechanism for stimulating the animal on recovery was also devised. This mechanism was so arranged as to be started by the recovery of the animal and stopped when the animal ceased to struggle and became immobile again.

Fortunately, a simple discovery rendered unnecessary this more or less elaborate set-up. The duration of tonic immobility in all cases has been found to be independent of the immobilizing stimulus. Any manipulation that produces the condition does so in an all-or-none fashion. While observing the properties of tonic immobility it was found that if, while lying on the back, one prevented the immobilized *Phrynosoma* from righting itself on recovery that it immediately became reimmobilized. This was demonstrated for all of the eleven horned "toads" in the laboratory at the time. If one but applied light pressure to the skin on the sides of the animals so as to prevent their turning over they relapsed after a brief struggle into immobility, *the same in all of its properties* to that produced by the usual sternal pressure.

It now only became necessary to fasten the animal on its back, in the thermostat, connect it with the writing lever of the kymograph, and have it make its own record of spontaneous recovery and immobilization. A holder was made for the lizards,

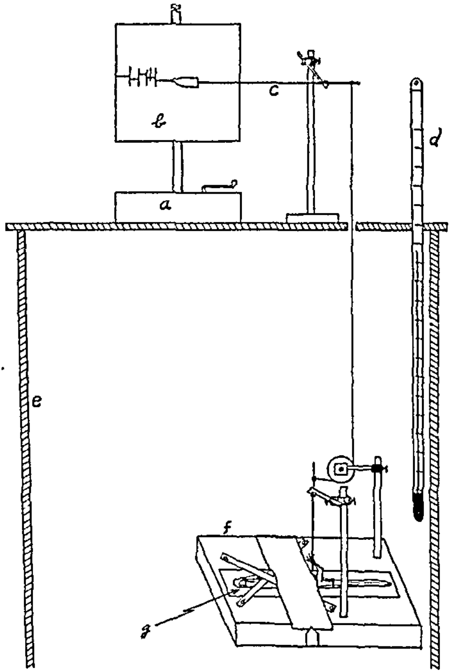

FIG. 1. Apparatus for obtaining records of spontaneous emergence from tonic immobility: *a*—kymograph, *b*—drum, *c*—writing lever, *d*—thermometer, *e*—wall of thermostat, *f*—animal holder, *g*—animal.

consisting of a block of wood with a depression gouged out of it. This depression was filled with paraffin and hollowed out in such a way that *Phrynosoma* would fill it when placed in it dorsally. In order to prevent escape, straps were placed

across the body of the animal, one running directly across from side to side and two others passing diagonally across the thorax. One of the lizard's legs was fastened by a strip of adhesive tape to a vertical lever supported by the holder block. A thread was fastened to this lever and after passing under a pulley was run upwards out of a hole in the top of the thermostat in which the lizard was placed. This thread was then directly connected to a writing lever, and in this way the movements of the animal's leg were recorded on a slowly revolving smoked drum.

It may be objected that the animal might fail to move the recording leg when it recovers. From many observations of the phenomenon, the recovery process has been observed to be constantly the same in certain particulars. It is always characterized in the cases of both *Anolis* and *Phrynosoma* by immediate struggles to turn the ventrum downward. This reaction on the part of the animal seems to be at a reflex level and is invariable. Moreover, the act of turning over requires the utilization of the legs and in all observed cases the legs are moved vigorously.

Although a number of kymograph records were obtained with *Phrynosoma* this animal was discarded for *Anolis carolinensis* owing to the excessively long periods of immobility occurring at intervals in the case of the former. The method developed for *Phrynosoma* obviated the difficulties of hyperexcitability formerly encountered with *Anolis* and at the same time the shorter periods of *Anolis* made it possible to obtain more data. It was found that the property of reimmobilization by preventing the animal from turning over is also characteristic of *Anolis*. Certain minor modifications of the animal holder were the only changes necessary in the foregoing method (see Fig. 1).

179 records of an approximate average duration of 4 hours each, and comprising 12,000 to 15,000 mobilizations were made over a period of 5 months. In all, six animals were used, four of which lived in a healthy condition throughout the experiments. Of the two that died one was killed by accident and the other died a "natural" death, but in both cases after a significant amount of data had been obtained.

The low temperature thermostat was adapted by the immersion of a cylindrical can 8 inches in diameter and 9 inches deep in the water bath. The animal holder was placed in this can and the thread connecting the leg with the writing arm was run out through a hole in the top. The kymograph was placed on a platform across the bath above the can and a calibrated thermometer was suspended inside to record the air temperature near the animal. The incubator set-up used for work above room temperature is diagrammed in Fig. 1.

The calibrated kymographs turned approximately once in 8 hours, corresponding to about $\frac{1}{2}$ mm. of record per minute. Individual animals were not tested twice on the same day but only after at least 24 hours in the terrarium. The records were taken as intervals of 2½°C. from 5° to 35°C. In the cases of the four surviving animals at least two and in some cases three and four runs were made at each of the temperature intervals.

III.

Fig. 2 is a sample set of records. The three numbers on the record at the left hand side reading from left to right designate the number of the run, the number of the animal, and the mean temperature.

The records show a rhythmical distribution of recovery periods in most cases, these rhythms in general shortening with rise of temperature. Certain other records, illustrated by the three sample records on the left side of Fig. 3, show a definite decrease in the number of recovery periods with time until finally the animal may remain continuously immobile for hours.

The data of Fig. 2 were all taken with the same animal and the effect of temperature on the duration of immobility is clear. The vertical marks on the record are caused by the movement of the leg connected with the writing lever. (Owing to the slowness of rotation of the drum, the few seconds that the animal is active during each recovery are not recorded, the up stroke representing a recovery and struggle period during which the pointer moves up and down in essentially the same track).

In computations made from the records the 1st hour of the run was not considered, in order that the temperature conditions should come *to equilibrium and the effect of general excitement, incident to starting* the experiment, should be minimized.

Run 48 at 5.9°C. demonstrates a long set of rhythms, the average duration, made from the last five, being 39 minutes. Run 74 at 10.7°C. shows a marked decrease in the duration of the rhythms, just one recovery period in each case marking the limit of the cycle (average = 12.3 minutes). In this case there is observed a characteristic, sudden cessation of recoveries at the end of several hours with only one or two struggles after irregular intervals. Run 167 at 14.5°C. is an excellent sample of the sort of result obtained at this temperature. Here are periods of immobility each representing a rhythm varying at remarkably regular intervals (mean = 4.5 minutes). This distribution remains constant after $3\frac{1}{2}$ hours of the experiment. Run 129 at 19.1°C. is of interest. In this case there occurs apparently a double rhythmical effect. The period of immobility which at 14.5°C. was 4.5 minutes long has now become reduced to 2 minutes while another

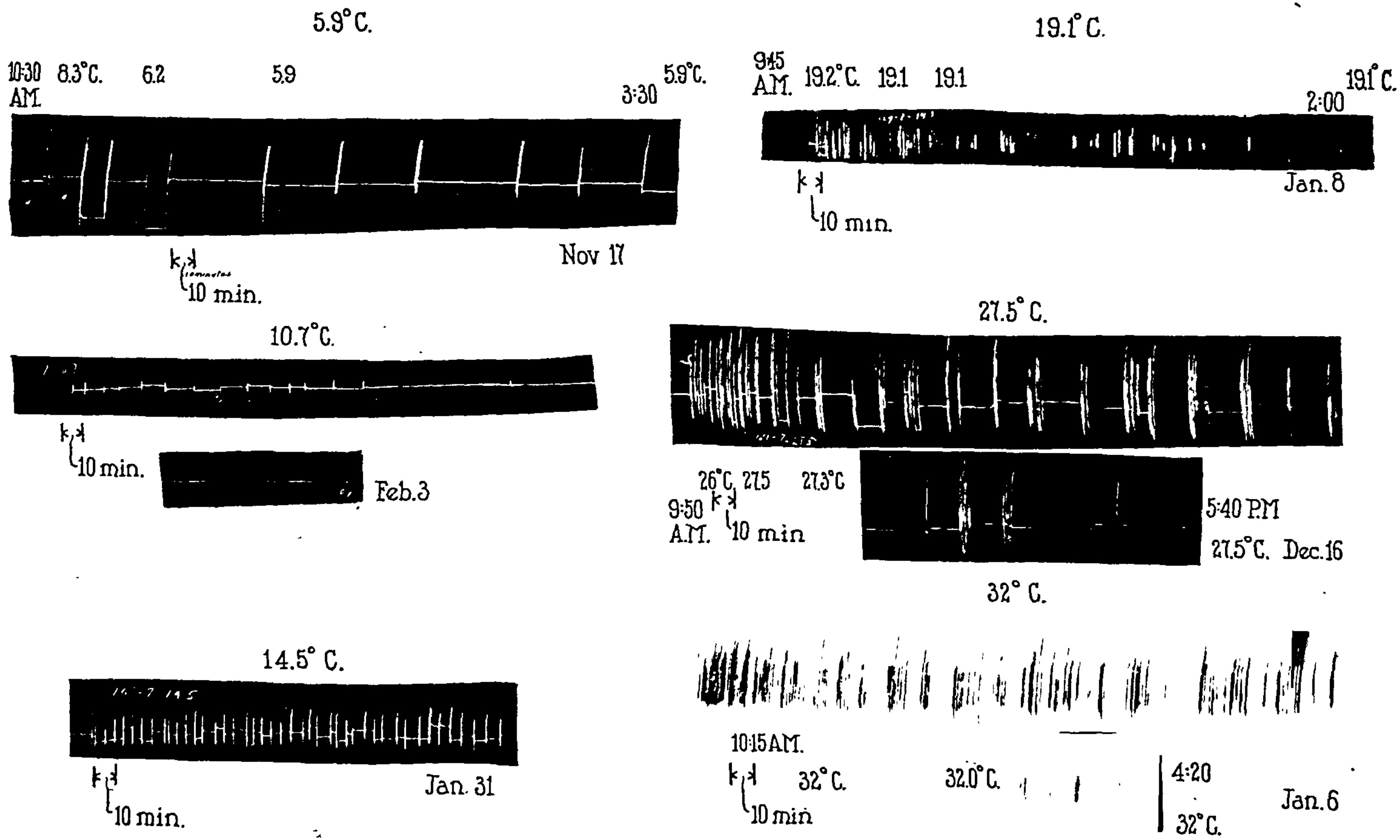

FIG. 2. Sample kymograph records from a single animal. The height of the vertical strokes of these records has no significance. The horizontal distances between strokes gives a direct measure of the duration of tonic immobility between spontaneous recovery and reimmobilization and succeeding spontaneous recovery and reimmobilization. The struggles of the animal actually occupy but a few seconds and so are only registered as a single up stroke on the slowly moving drum. The records illustrate the effect of temperature on the duration of immobility. The three numbers at the left of the records are respectively the number of the experiment, the number of the animal, and the temperature.

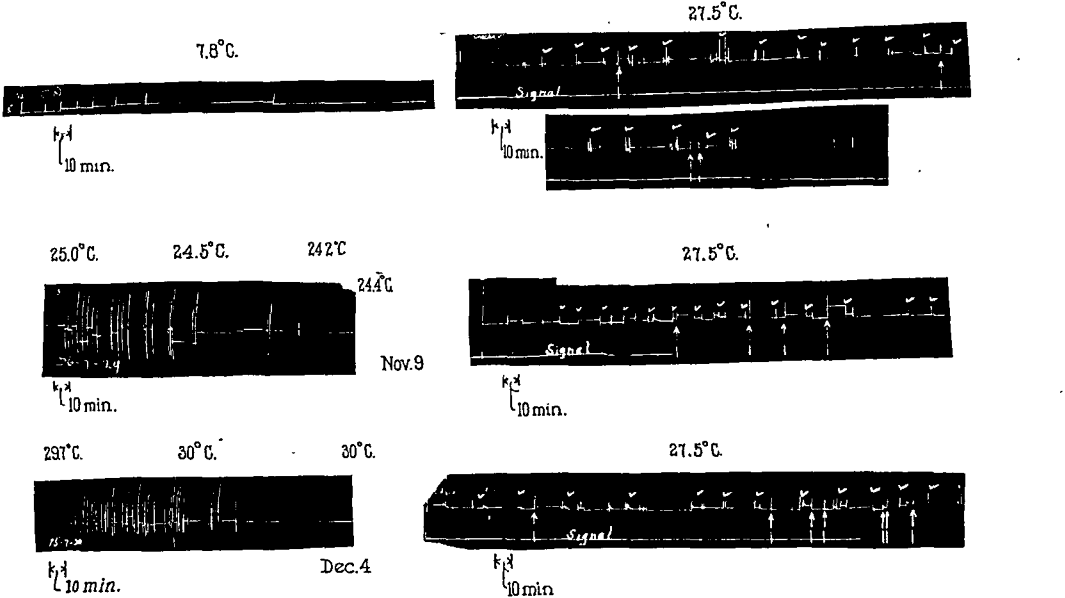

FIG. 3. The three sample records on the left of this figure show a definite decrease of frequency of recovery with time.

The three records on the right demonstrate the essential functional independence of the rhythms. The check (V) marks indicate rhythms, and the signal below marks the place of forced recovery of the animal by shocking it with an inductorium. It will be seen that forced recovery has no effect on the rhythm or subsequent rhythms, the animal becoming reimmobilized and remaining so until its normal period of recovery.

rhythm of 12 minutes duration seems to be superposed, as it were, on the original shorter rhythm. In the case of all animals examined this additional rhythm was found to occur at approximately 20°C. and to continue in more pronounced form with rise of temperature to about 32°C. Run 101 at 27.5°C. and Run 123 at 32°C. demonstrate the double rhythmical effect which is most pronounced at 27°C. In these cases one finds a fundamental unit of immobility time (2 minutes to $\frac{1}{4}$ minute), shortening with rise of temperature, with superposed upon this a well marked longer rhythmical cycle. As will be seen presently, this longer rhythm also shortens statistically with rise of temperature.

Runs 172, 36, and 75 of Fig. 3 demonstrate the general exhaustion of the ability of the animal to recover from the immobile state. All of the samples of Figs. 2 and 3 are chosen because of the definiteness of the phenomena. In many cases the rhythms are not as obvious as in these and practice is necessary to enable one to identify them. The other records of Fig. 3 serve to demonstrate a point of interest. It was desired to learn whether tonic immobility was caused by the release of some mechanism independently at each inducing stimulus, or whether the initial stimulus in a sequence releases a series of events which proceeds independently of succeeding stimuli. The latter had been found to be essentially the case by Crozier in his work with arthropods. In order to test this with *Anolis* an animal was "awakened" at various stages in the immobility period and the effect on subsequent periods was observed. The right-hand records of Fig. 3 illustrate the results. The lower line on these records was made by a signal magnet which was connected in series with an inductorium. At intervals during the rhythms as indicated by the signal the animal was shocked until it recovered activity and struggled to escape. During the period of stimulation the thermostat was opened briefly and the animal watched to make certain that it had fully recovered and that the mark on the record was not merely made by a reflex twitch in response to the shock. The rhythms in the records have been accentuated for convenience by check marks. It is apparent that the stimulus merely broke in upon an already functioning process which had been started by the initial stimulus. The forced recovery, even when the animal was aroused twice in the same cycle, has no effect on the general rhythm.

It was desired to make certain that the internal temperatures of the animals were equal to those of their surroundings after adequate exposure, and to discover any possible increase in temperature during the recovery struggle. A calibrated thermopile was inserted in the cloaca of one of the animals and the preparation was placed in a thermostat, a thermometer near the animal giving the temperature of

FIG. 4. This figure with Figs. 5, 6, 7, and 8 illustrates the effect of temperature on the duration of the rhythms of tonic immobility of *Anolis carolinensis.* The data were plotted according to the Arrhenius equation and μ was calculated from the slopes of the lines. The points in general fall in bands between the parallel lines. The upper and steeper curve represents the rhythm in evidence at 5.9°C. (Fig. 2) which decreases throughout the temperature range. The lower curve corresponds to the secondary rhythm which becomes evident at approximately 20°C.

The lattitude of variation of the data is large. This may be due to the fact that the experiments were made over a 5 months period while with most temperature work the data for one plot are taken within 24 hours. Moreover, in these cases one is dealing with an obviously complex process and points lying between the normal latitudes of variation may be accounted for by a confusion of the two rhythmical processes concerned. In the case of this figure the points lying between the bands were all obtained during approximately the first month of the animal's life in the laboratory and may possibly be correlated with the age of the animal.

Shaded points correspond to averages of at least five rhythms.

the surrounding air. It was found that the temperature of the animal was appreciably below that of its environment (temperature range of 8° to 30°C.) the difference being as great as 0.4°C. at 8°C. and decreasing with rise of temperature. This negative discrepancy is puzzling but may be due to evaporation of moisture with respiration. It is more or less consistent with similar results found by Buxton (1924) in the cases of certain desert insects.

No appreciable internal temperature change occurred when the animal struggled during the recovery periods.

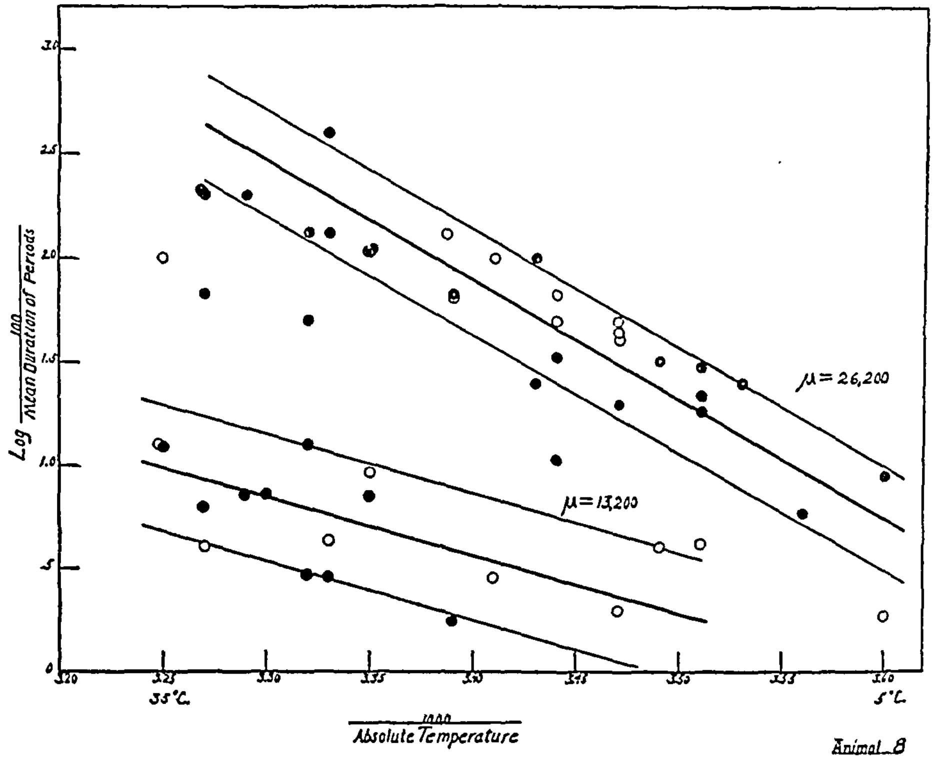

FIG. 5. Plot according to the Arrhenius equation illustrating the effect of temperature on the duration of tonic immobility for *Anolis carolinensis*.

IV.

The Arrhenius equation was applied to the data of those records showing rhythmical distributions of immobility periods as demonstrated in Fig. 2. The mean durations of immobility are shown plotted as a function of the absolute temperatures in Figs. 4, 5, 6, 7, and 8.

In these figures two distinct distributions of points with characteris-

tic μ values may be seen. These distributions are enclosed between parallel lines and an average central line is drawn through the field of points. This is permissable since the points do not merely form a fan-shaped band but group themselves in the indicated distributions (*cf.* Crozier and Stier, 1927). The solid points represent weighted averages of not less than five distinct rhythms during the experiment; the open circles correspond to less certain averages. The mean probable error of the durations of rhythm of some 28 experiments on one animal, throughout the temperature range, is $\pm$ 4.9 per cent.

The latitude of variation of the plotted data may seem large, but in later sections an attempt will be made to account for this scatter in terms of a theory of the mechanism of tonic immobility.

The results of the Arrhenius equation plots may be summarized in the following table:

Animal	μ_1, calories	μ_2, calories
7	7,330	31,400
8	13,200	26,200
11	*	35,000
13	9,210	31,800
14	*	30,700
Arithmetic mean..........	9,913	31,020
Probable error of mean......	$\pm$1,310	$\pm$868

* In the case of Animal 11 the data was insufficient for a determination of μ. With Animal 14 the scatter of points is too great to permit an assignment of a "μ" value.

V.

It is necessary to consider the type of record illustrated on the left in Fig. 3, in which there is a progressive increase in the durations of immobility. Many records show this phenomenon alone, but a few show a combination of rhythmicity with progressive prolongation of immobility.

Plots were made of the logarithm of the frequency of recovery per hour against the total time from the beginning of the experiment and in most cases surprisingly good linear functions were obtained. A sample curve of this sort is illustrated by Fig. 9.

The values of R, the slope of the lines, were plotted as a function of the temperature. This was done by expressing the values of R for each temperature as a percentage of the mean value of R for the particular animal, and on this basis averaging data from all of the animals together in one plot. Fig. 10 is the result, averaged for 5° intervals.

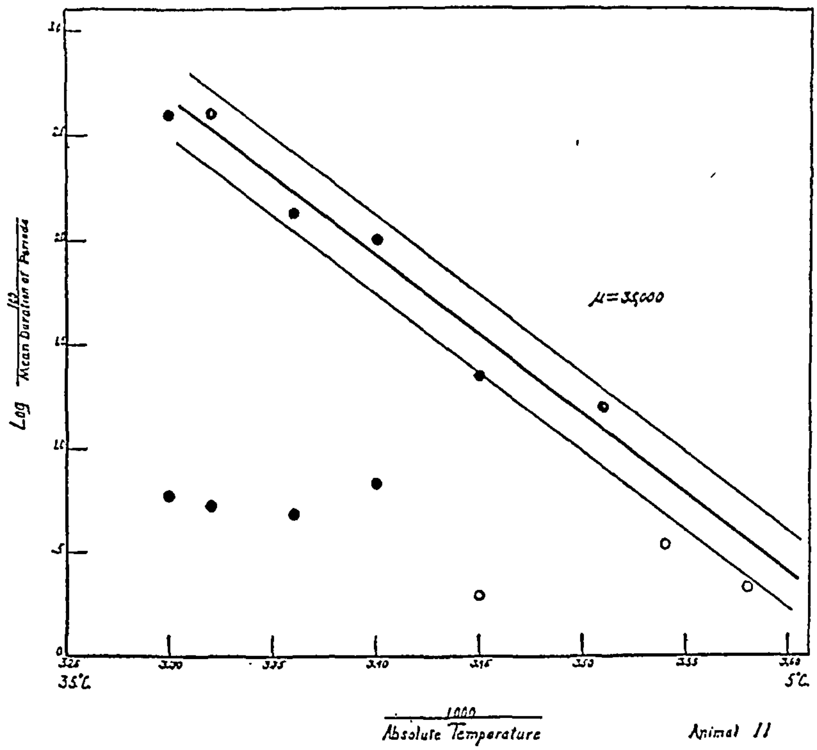

FIG. 6. Plot showing temperature effect on the immobility periods of *Anolis carolinensis*. In this case the animal died before enough data were obtained for a calculation of the lower μ.

This plot clearly shows that the effect of temperature on the function in question is substantially negligible, the computed value of Q_{10} being 1.04.

A value for Q_{10} of 1.04 is essentially not that characteristic for a chemical process (Q_{10} = 2 to 3). The value of Q_{10} for diffusion processes in solution is of the order of magnitude of 1.0 to 1.3. Fig. 10 makes it seem likely that, in the cases under consideration, some physical event is determinative.

VI.

The application of the Arrhenius equation to those records which show a rhythmical sequence of successive immobility periods suggests a basic chemical mechanism of control for the duration of tonic immobility. This is further borne out by the fact that if the animal is forced to recover in the midst of a period and is then reimmobilized (*cf.* Fig. 3, right) it remains immobile until that time when it would have recovered had the period not been interrupted. To account for

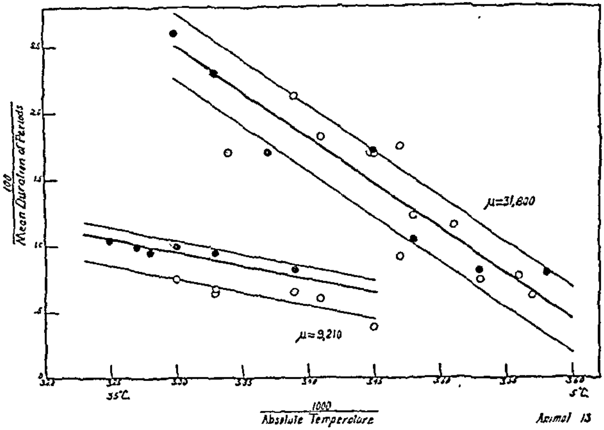

FIG. 7. This is perhaps the clearest illustration of the effect of temperature on the duration of tonic immobility of a single *Anolis*.

this last fact and for the suddenness and completeness of recovery the presence of a definite amount of substance inhibitory to the "higher centers" but allowing impulses from internuncial neurones at the "tonic centers" to pass to the muscles may be assumed. Accordingly the animal remains immobile until the decomposing substance, excreted perhaps from an endocrine organ in response to the immobilizing stimulus, reaches a definite threshold level when the animal recovers. This threshold is qualitatively suggested by inspection of the rhythmic kymograph records.

It was originally suggested, to account for the effect of temperature on the periods of immobility (Hoagland, 1927) that two independent autacoids might be excreted from endocrine organs in response to the immobilizing stimulus. One of these was regarded as active over a range of 5° to 35°C. and the others active from approximately 20° to 35°C. In the case of *Anolis* immobilization usually results after a struggle and, if this hypothesis is correct, one or both of the autacoids might be released as a result of the struggle. It was also originally

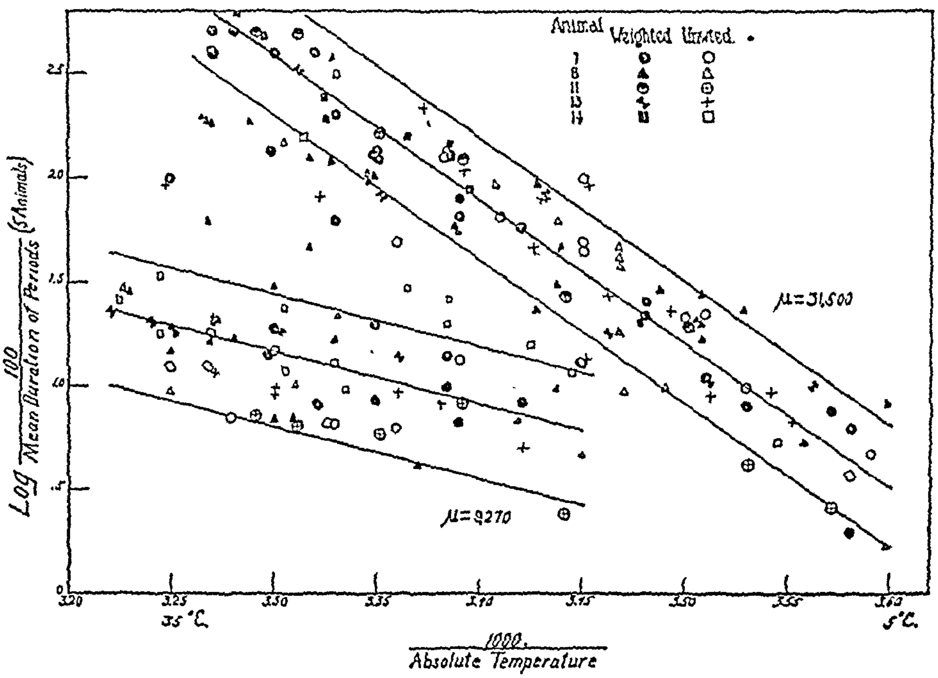

FIG. 8. Plot showing distribution of points for five animals according to the Arrhenius equation.

thought that the substances assumed, α and β, were not themselves inhibitors but that they decomposed to Inhibitors A and B respectively, in this way giving the evidence of chemical control shown by the Arrhenius equation plots. This assumption, in the light of more recent experiments, seems unnecessary. It is more probable that the inhibitors are the initial substances, α and β, which decompose to physiologically inactive substances, A' and B'.

To account for those records in which a progressive increase in the

durations of immobility are manifested (*cf.* Fig. 2) and which show the temperature effect characteristic of a physical change (*cf.* Figs. 9, 10), a diffusion process may be assumed controlling the amount of available catalyst in the decomposition of α and β. This is consistent with the evidence of a logarithmic accumulation of the hypothetical inhibitory

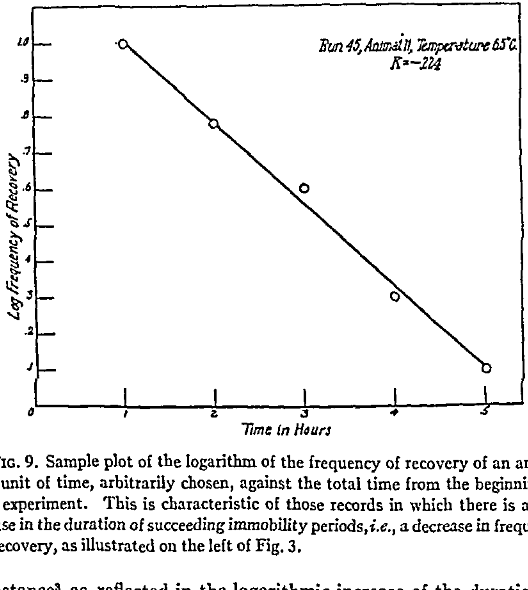

FIG. 9. Sample plot of the logarithm of the frequency of recovery of an animal per unit of time, arbitrarily chosen, against the total time from the beginning of the experiment. This is characteristic of those records in which there is an increase in the duration of succeeding immobility periods, *i.e.*, a decrease in frequency of recovery, as illustrated on the left of Fig. 3.

substance[3] as reflected in the logarithmic increase of the duration of immobilization. Recently, experiments have been made to ascertain

[3] It was observed in the course of the experiments that certain irregularities in the duration of tonic immobility periods served as an index of the approaching death of the animal. This was true for both *Phrynosoma* and *Anolis*, and it was also true for the isopods with which Crozier worked. It was found possible in several cases to predict the death of an apparently normal animal as much as 3 days in advance by irregularities in the rhythms. This fact suggests a possible relationship between the durations of immobility and metabolism.

the specific nature of the inhibitory "hormones" which might thus be involved. The injection of small amounts of adrenalin, in excess of a definite threshold amount, is found to prolong markedly the durations of tonic immobility in *Anolis*. The adrenalin is injected intraperitoneally (0.1 cc.), after diluting with Ringer's solution to concentrations varying with the experiments between 0.5:100,000 and 5.0:100,000. Fig. 11 shows typical samples of experiments demon-

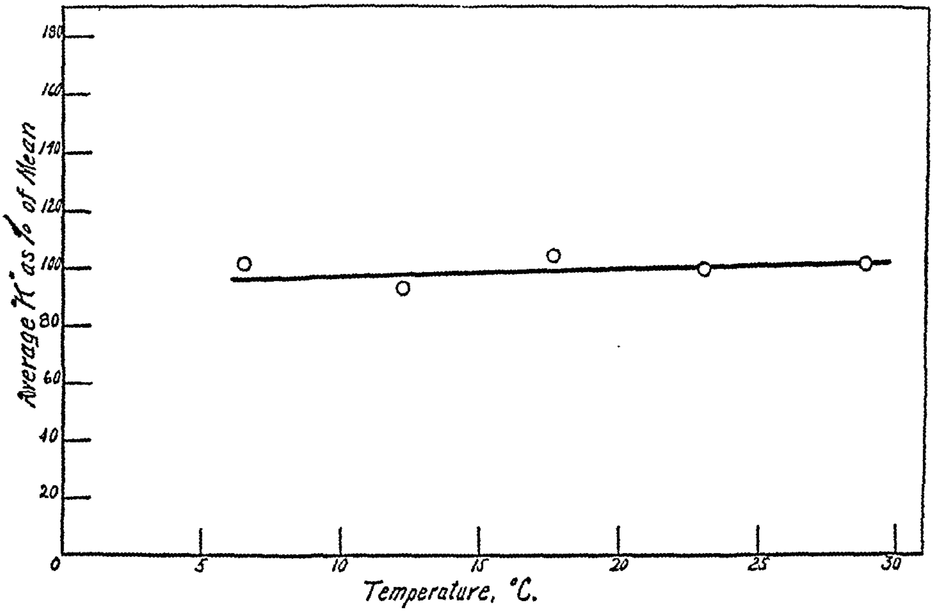

FIG. 10. This figure illustrates the effect of temperature on the frequency of recovery from tonic immobility in those cases in which the frequency decreases progressively with time from the beginning of the run. The constant K as ordinate is the slope of the lines measured from the frequency of recovery *vs.* time plots illustrated by Fig. 9. Each point of this figure represents an average of approximately five such slopes as that illustrated in Fig. 9.

strating the effect of adrenalin. Controls injected with 0.1 cc. of Ringer's solution show no prolongation of immobility. The injections were made with the animal in the recording mechanism at 27.5°C. after 1 hour from the beginning of the experiment. The animal was not removed from the holder or otherwise disturbed at the time of injection. Fig. 12 represents the *number of normal spontaneous recoveries missed* after adrenalin injection plotted as a function of the *amount of adrenalin* injected. The shaded circles are weighted aver-

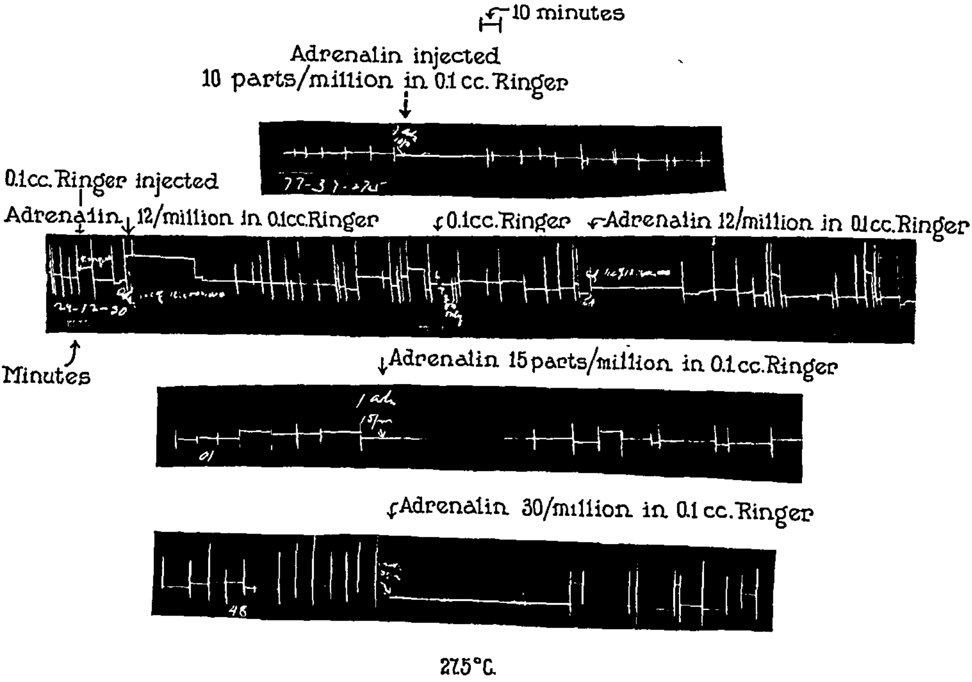

FIG. 11. Sample records from 80 experiments showing typical effects of adrenalin in Ringer's solution injected intraperitoneally in causing *Anolis* to miss normal recoveries. 0.1 cc. Ringer's solution without adrenalin does not *prolong tonic immobility* as may be seen in the second record.

ages of 80 experiments with many animals,[4] the unshaded circles are unweighted averages, and the crosses are points for one individual animal. Probable errors are indicated on the plot by lines through the points perpendicular to the abscissa. The curve may be a measure of the rate at which the adrenalin becomes inactive, since the number of periods missed is measured in time units; or it may de-

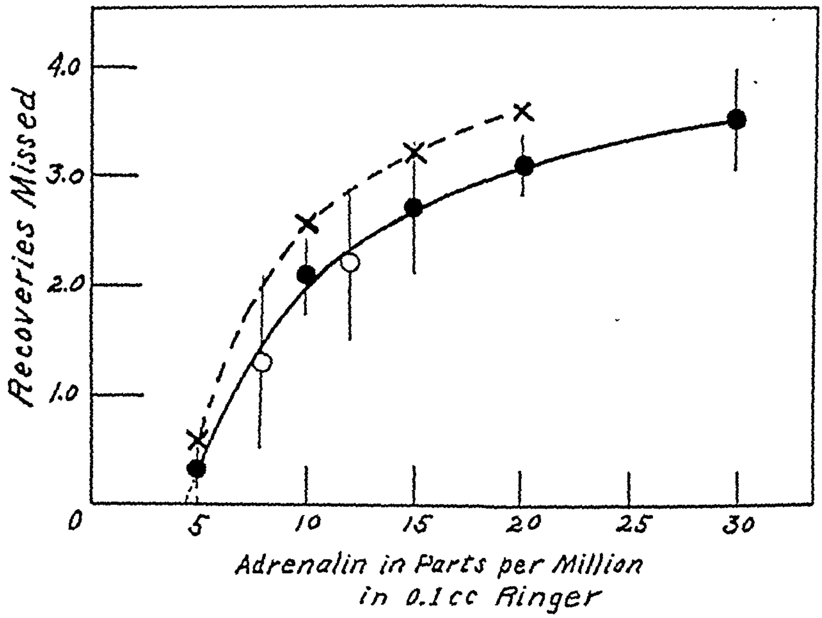

FIG. 12. Curve relating dose of adrenalin to number of normal spontaneous recoveries missed. The shaded circles are weighted averages of 80 experiments with many animals and the unshaded circles are unweighted averages. The crosses are points for one animal. The probable errors are indicated by lines through the average points perpendicular to the abscissa. A threshold for injected adrenalin is indicated by extrapolation. Controls injected with 0.1 cc. of Ringer's solution show no prolongation of periods of tonic immobility, *i.e.*, no normal recoveries are missed.

pend upon the diffusion of adrenalin from the body cavity. Moreover, the threshold previously suggested is found in the case of injected adrenalin by extrapolation of the curve to the abscissa. For the conditions of these experiments it is approximately four parts per million of adrenalin in 0.1 cc. of Ringer's solution. A lower and pos-

[4] Only those experiments in which definite rhythmicity of periods occur have been treated.

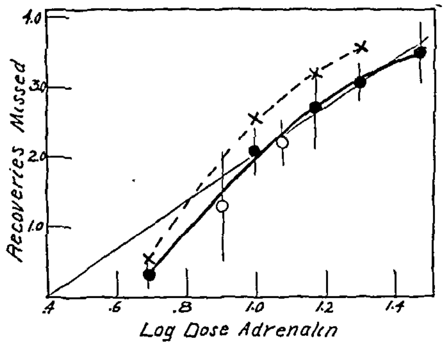

FIG. 13. Plot of logarithm of dose of adrenalin against the number of periods missed. The points are designated as in Fig. 12. The thin line cutting the abscissa at 0.4 corresponds to a threshold concentration of 2.5 parts per million of adrenalin in 0.1 cc. Ringer's solution. This line passes through the higher, more reliable points as described in the text.

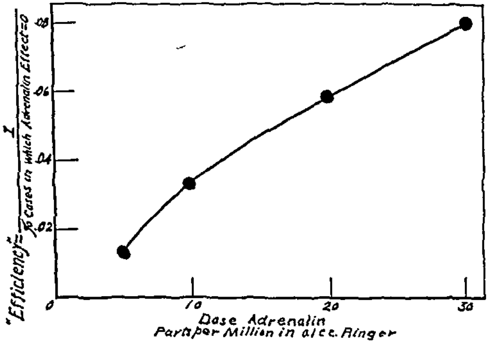

FIG. 14. Plot for principally weighted points of "efficiency" of adrenalin in prolonging tonic immobility against the dose of adrenalin in parts per million in 0.1 cc. Ringer's solution. The "efficiency" is measured by the reciprocal of the per cent of experiments at a given dosage in which the prolonging effect of adrenalin is zero.

sibly more significant apparent value for this threshold will be discussed presently.

Fig. 13 is a plot of the logarithm of the dose of adrenalin against the number of recoveries missed. The line is slightly curved but is closer to a straight line than any other simple curve which "makes sense" in terms of chemical dynamics. The departure from the logarithmic may readily be accounted for in terms of the rather large probable errors and probably has no theoretical significance.

It was found that sometimes adrenalin has no effect in prolonging tonic immobility. A given dose which, on the average, may cause the animal to miss several recovery periods, may sometimes have no effect at all, and at other times may have two- or threefold the average effect. The occurrence of the "zero" effects of adrenalin is more pronounced at low than at high concentrations. Fig. 14 shows the "efficiency" of adrenalin in prolonging tonic immobility plotted against the dose of adrenalin for the four principally weighted doses; the efficiency is here measured by the reciprocal of the per cent of experiments in which the effect of adrenalin is zero.[5] With the exception of the point at five parts per million the line is substantially straight indicating a direct proportionality between the amount of injected adrenalin and its efficiency. Since the lowest point on the curve seems to be an exception to this relationship, the results at higher concentrations may be regarded as more reliable. A line through these higher points (Fig. 13) cuts the abscissa at 0.4. The antilog of this is 2.5, which is probably a more significant threshold value (parts per million in 0.1 Ringer) for adrenalin than that obtained by the extrapolation of the curve of Fig. 12. This value corresponds to about 0.1 mg. adrenalin per kilo body weight.

The logarithmic function (Figs. 12 and 13) is what one would expect if adrenalin were decomposing according to a first order chemical process. The oxidation of adrenalin in the presence of excess oxygen

[5] The weighted point corresponding to the concentration of 15 parts per million is left out of Fig. 14. The per cent of cases in which the adrenalin effect is zero is abnormally high for this point. This abnormality may be explained by the fact that the data at this concentration were taken from two groups of animals of different ages and sizes at intervals of several months. The probable error for this point is also high in spite of many experiments. Because of this lack of homogeneity of material the point has been excluded.

would give such a function. The temperature analysis of tonic immobility, it will be remembered, yielded two values of the critical thermal increment, μ, tentatively indicative of respiratory (oxidative) processes. It therefore seemed probable that if internally secreted adrenalin were one of the assumed autacoids which becomes inactivated through oxidation, that adrenalin first oxidized in the air and then injected into the animal should have no effect on tonic immobility. This was found to be the case. Adrenalin allowed to stand in the air until oxidized, as indicated by the solution turning pink, was found to have no effect whatever on tonic immobility.

Forced recovery in the midst of a period that has been lengthened by adrenalin injection shows the same result as is shown by forced recovery during a normal period (*cf.* Fig. 3). The animal if re-immobilized remains immobile for the remainder of the duration characteristic of the particular dose of adrenalin.

While it is possible that adrenalin, on the basis of this evidence, may be one of the hypothetical autacoids which prolongs tonic immobility when present above a threshold concentration, this is not certain. The injection of ergotamine, in the case of mammals, is known to paralyze the endings of the sympathetic nervous system. If it has a similar effect on lizards and if adrenalin acts peripherally in producing tonic immobility one would expect that tonic immobility would be abolished by this drug. Exactly the opposite effect results.[6] Since it is not certain how ergotamine affects the sympathetic nervous system of *Anolis*, this experiment is not in itself determinative. The fact that spinal reflexes may be elicited during tonic immobility is a strong argument that the effect is central rather than peripheral, since if the inhibiting autacoid acted peripherally one would expect it to block spinal reflexes. If, in this case, adrenalin acts *centrally*, any sympathetic paralysis due to ergotamine would not necessarily shorten or abolish tonic immobility. The prolonging effects of ergotamine

[6] Not only does ergotamine prolong tonic immobility but an excessively large injection of Ringer's solution (0.2 cc.) may also prolong it slightly. These injections along with that of adrenalin may be assumed to increase the blood pressure. Amyl nitrite vapor, on the other hand, which presumably reduces blood pressure, abolishes immobility in *Anolis*, causing the animal to struggle violently. Both ergotamine and amyl nitrite are highly toxic and little can be concluded regarding isolated pressor and depressor effects in these cases.

may be due to general toxic effects which have nothing to do with the specific relations of adrenalin and the sympathetic nervous system to the mechanism controlling tonic immobility.

While the effect of temperature on the durations of immobility, the continuance of the rhythm after the animal has been aroused and reimmobilized, and the adrenalin effects, all point to a hormone (possibly adrenalin) controlling tonic immobility, there are other facts to be considered that at first seem difficult to reconcile with this general picture. The onset of the condition is very sudden. It follows immediately, with birds and mammals, on merely turning them quickly dorsum downward. This is hard to explain in terms of a process *initiated* by a hormone. Moreover, it is noteworthy that the mere injection of adrenalin does not produce tonic immobility; it only prolongs the state if it is already existing.[7]

The "shocking" effect on the centers of reflex tonus in the bulb and cord of suddenly removing an animal from the substratum and overturning it, or by allowing it to struggle on its back with limbs in the air so that normal afferent stimuli from the feet and from the stretched muscles are abolished, must be considerable. At all times the muscles controlling body posture are in a state of tonus determined by nicely graded reflexes afferently controlled by the amount of stretch of the muscles and by the mechanisms of the internal ear (*cf.* Cobb, 1925; Magnus, 1924). The sudden overturning of an animal must result in a decided unbalancing, so to speak, of the central machinery of reflex tonus. In a very brief interval the tensions of most of the large muscles are changed, which may be understood to result in a disorganized volley of afferent impulses sent to the internuncial neurones at the tonus centers. Since the animal's limbs are removed from the substratum there is no possibility of rapid readjustment by way of normal afferent stimuli and it seems not unlikely that such a condition might result in a promiscuous discharge of the tonus centers effecting an immediate "locking" of the entire musculature.

Such a violent discharge of a group of internuncial neurones might also be expected to influence the adrenal glands. Adrenalin might,

[7] In some experiments *Anolis* was injected with adrenalin but not immobilized until some minutes later. In these cases the duration of immobilization was also found to be increased.

therefore, be excreted in excessive amounts and, once in the circulation, act as we know that injected adrenalin does to prolong the state of tonic immobility. According to this hypothesis, the hormone in question does not in itself initiate tonic immobility but serves to maintain the predominance of those tonic impulses already passing and to prevent impulses from higher centers from gaining access to the final common paths to the muscles. In the case of arthropods a similar mechanism may apply. Arthropods are often readily immobilized by merely lifting them from the substratum thereby producing pronounced changes in the stretch of the limb muscles. While it is not likely that adrenalin is the autacoid involved in these cases there is good evidence, as Crozier has pointed out, to suppose that some inhibitory substance controls the durations of immobility.

In addition to the question of the *mechanism* of tonic immobility, the condition itself offers opportunity for the study of tonus in skeletal muscle in the intact organism. The immobile animal resembles in many respects a decerebrate preparation. Certain aspects of deep reflexes are conspicuous, and experiments are in progress to measure under these conditions the frequency of discharge of certain stretch reflexes of *Phrynosoma* as a function of the tension in the muscles.

SUMMARY.

1. The durations of successive periods of induced tonic immobility in the lizard *Anolis carolinensis* was examined as a function of temperature. An automatic recording method was employed and observations were made of 12,000 to 15,000 immobilizations with six animals over a temperature range of 5° to 35°C. during 5 months.

2. The durations of the immobile periods were found to vary rhythmically in most cases.

The reciprocal of the duration of the rhythm, *i.e.*, the rate of change of the process underlying the rhythms, when plotted as a function of temperature according to the Arrhenius equation show distributions of points in two straight line groups. One of these groups or bands of points extends throughout the entire temperature range with a temperature characteristic of approximately $\mu = 31{,}000$ calories, and the other covers the range of 20° to 35°C. with μ equal to approximately 9,000 calories.

3. The initial stimulus in a series of inductions of immobility appears to set off a mechanism which determines the duration of the state of quiescence. Succeeding forced recoveries seem to have no effect on the normal duration of the rhythm.

4. These results are interpreted by assuming the release, through reflex stimulation, of hormonal substances, one effective between 5° and 35°C. and the other effective between 20° and 35°C. These substances are assumed to act as selective inhibitors of impulses from so called "higher centers," allowing impulses from tonic centers to pass to the muscles.

5. In some experiments a progressive lengthening in successively induced periods of immobility was observed. The logarithm of the frequency of recovery when plotted against time in most of these cases (*i.e.*, except for a few in which irregularities occurred) gave a linear function of negative slope which was substantially unaffected by temperature. In these cases it is assumed that a diffusion process is controlling the amount of available *A* substance.

6. The results are similar to those obtained by Crozier with *Cylisticus convexus*. The duration of tonic immobility seems to be maintained in both arthropod and vertebrate by the chemical activity of "hormonal" selective inhibitors. The details of the mechanisms differ, but there is basic similarity.

7. Injections of small amounts of adrenalin above a threshold value are found to prolong the durations of tonic immobility of *Anolis*, by an amount which is a logarithmic function of the "dose." It is possible that internally secreted adrenalin, above a threshold amount, may be involved in the maintenance of tonic immobility.

8. The *production* of tonic immobility reflexly is a problem distinct from that of the *duration* of immobility. It is suggested that the onset may be induced by "shock" to the centers of reflex tonus causing promiscuous discharge of these centers with accompanying inhibition of the higher centers. Such a condition may result when an animal is suddenly lifted from the substratum and overturned, or when, as in the case of *Anolis*, it struggles with dorsum down. This reaction of the "tonic centers" may at the same time lead to discharge of the adrenal glands by way of their spinal connections thus prolonging the state.

The writer wishes to express his thanks to Professor W. J. Crozier, in whose laboratory the work was carried out, for his many helpful suggestions.

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THE MECHANISM OF THE INFLAMMATORY PROCESS.

III. Electrophoretic Migration of Inert Particles and Blood Cells in Gelatin Sols and Gels with Reference to Leucocyte Emigration through the Capillary Wall.*

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INTRODUCTION.

In the first paper of this series (1, 2) it has been shown that the potential differences probably existing between injured and relatively normal tissues are of a sufficient order of magnitude to account for the migration of leucocytes through connective tissue to a point of injury. The migration of a white cell through a gel-like medium was not then considered in detail.

The data presented also demonstrated that in plasma and in serum cataphoresis of white and red cells obey the Helmhotz-Lamb equation,

$$V = \frac{H\,D\,\zeta}{4\,\pi\,\eta} \tag{1}$$

V = velocity of particle, D = dielectric constant of the medium, ζ = electrokinetic potential, η = viscosity of the medium (all units c.g.s. electrostatic), since for different values of H, it was found that

$$V = k\,H. \tag{2}$$

That is, the speed of cataphoretic migration was proportional to the applied difference of potential. This was noteworthy because of the high concentrations of proteins present in the solutions used.

Opposing the migration of a leucocyte to a point of injury is a gel-like system, the capillary wall, whose components would probably

* The researches reported here were for the most part completed during a tenure of Fellowship in Medicine of the National Research Council.

Part of the data was presented at the June (1928) meeting of the Sixth Colloid Chemistry Symposium in Toronto.

show a type of flow that would not obey Poiseuille's law. The flow of serum and plasma follows Poiseuille's law (3, 4). Gelatin sols, which do not follow Poiseuille's law, are said to have "elasticity"[1] or plastic flow. For small stresses the fluid changes its shape without flowing. Upon removal of the stress the system resumes its original form and position. Upon increasing the stress, a flow takes place. With slight stresses the amount of this flow is less than the amount expected from the proportional increase in stress. Only when the stress is sufficiently great is the flow independent of the rate of shear, and it is only then that the true viscosity, η, of the fluid is measured in viscosimeters of the Hess type (3, 5, 6). The apparent viscosity, η', as compared with the true viscosity is a measure of the flow of the system only for a given stress.

It is evident from the data previously given for serum and plasma (1, 2) that, although one would expect the tissue fluids of the interfibrillar spaces of connective tissue to have viscosities which could not appreciably hinder the migration of a leucocyte wandering cataphoretically, the presence of the gel-like structure of the capillary wall introduces several problems for discussion:

1. How does the presence of an "elastic" medium influence the cataphoretic migration of microscopic particles (*e.g.*, a soft gelatin gel or sol)?

2. What is the influence of an "elastic" medium on the migration of microscopic particles where the migration is due to a force other than a difference of potential?

3. What is the influence of solid strands, like those contained in a fresh fibrin gel, on leucocyte cataphoresis?

Method.

The majority of the experiments described in this communication were carried out in a one-piece glass cataphoresis cell quite similar in operation to the three-piece cell described by Northrop and Kunitz (7). Cu-$CuSO_4$-agar non-polarizable electrodes were found more convenient. Occasionally the cemented cell described by Freundlich and Abramson (2) was used. This cell is a modification of that described originally by Northrop. The potential differences applied were great

[1] "Elasticity" is here used as a translation of the work "Elasticitat" appearing so frequently in the German literature, in discussions of plastic flow.

enough to give an easily measured velocity without heating effects. This depends, of course upon the dimensions of the cell and the conductivity of the solution.

Agfa Lichtfilter gelatin was employed. In making up the gelatin sols, horse serum and gelatin were heated to 45°. Blood cells and quartz particles in suspension in another sample of unheated serum were added. The suspension was then cooled to room temperature, the electrophoresis cell was filled at once and measurements were started. During the first measurements the particles settled out of the sol. The cell must therefore be immediately turned upside down to allow the particles to be redistributed in the medium. The gel forms more or less slowly, the speed depending on the concentration; the cells thereafter remain suspended at practically the same level, thus making their migration simple to follow.

The measurements were made at room temperatures, but differences in room temperature make some difference in the consistency of the gel formed. Consequently in the tables ½ hour gel means that this gel consistency was generally obtained after this time but sometimes required more or less time. The point is, that *all* the changes must be simultaneously observed and checked.

TABLE I.

The Influence of the Gel Formation on the Cataphoretic Velocity of Inert Particles. See Figs. 1 and 2 for viscosity measurements.

Age of the sol or gel	Character of medium	Velocity
hrs.		*μ per sec. volt per cm.*
0.5	Sol	0.36
1.0	Sol	0.35
2.0	"Elastic" sol	0.35
3.0	"Elastic" sol	0.34
3.5	Soft gel	0.33
5.0	Soft gel	0.36

Cataphoresis of Certain Inert Particles in Gelatin Gels.

The electrophoresis of particles of quartz, of zinc and of silver as well as air bubbles from 1.0 to 50.0μ in diameter were studied in 1.0 per cent gelatin sols and gels (2). These inert particles are immediately surrounded by an adsorbed sheath of the protein and migrate through the gel with the same speed as the micells.

It is well known that freshly prepared gelatin solutions of this concentration show practically no "elasticity." Shortly after preparation, however, the properties of "elastic" fluids manifest themselves. Simultaneous measurements of cataphoresis of the particles and of η' and η were made (3).

Table I shows that the cataphoretic velocities of the particles remained constant during gel formation regardless of the increasing stiffness of the gel. That is, cataphoretic migration in such a soft (1 per cent) gelatin gel is independent of the apparent viscosity. Since the cataphoretic velocity is inversely proportional to the viscosity, it seemed that the true viscosity remained constant during gelation.

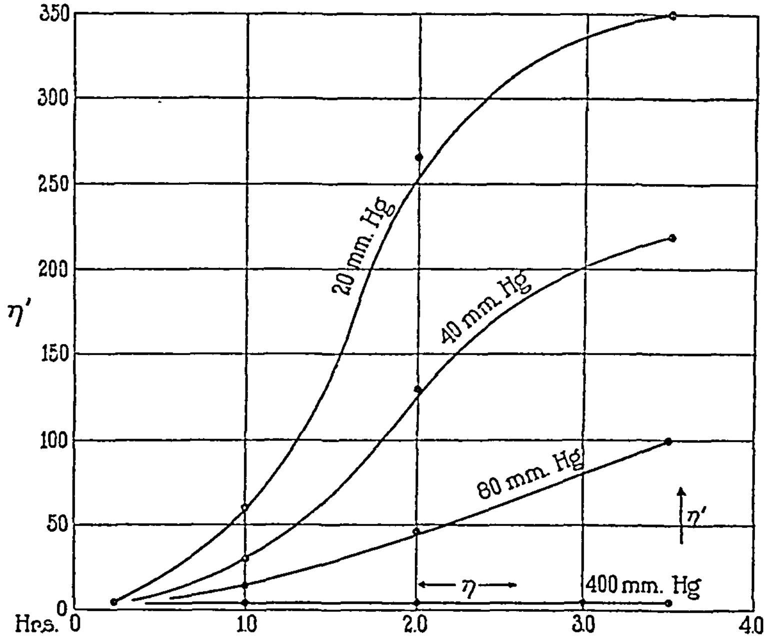

FIG. 1. This series of curves shows that with the ageing of a 1 per cent gelatin sol the viscosity, η, remains practically constant, as shown by the measurements made at 400 mm. Hg. pressure. A comparative enormous rise in η', the apparent viscosity, however occurs as shown by the measurements at lower pressures. Similar curves have been obtained from a variety of other "elastic" sols and gels. The mm. Hg refer to the pressure with which the solution was forced through the Hess viscosimeter. In this type of instrument the rate of shear is not proportional to the pressure.

In making the initial viscosity measurements (15 min. sol) in the Hess viscosimeter, a suitable sample of the solution was forced from the graduated portion into the second portion by a pressure of 20—mm. Hg. The time of flow was measured. The fluid was then sucked back into the first chamber of the viscosimeter and further measurements were made in succession at higher pressures (40 mm., 80 mm., 400 mm.). The apparent viscosity, η', is the elapsed time for the flow of a given volume divided by the time that a flow of a similar volume of water would

take at the same pressure. The viscosimeter and solution contained therein were allowed to stand a given period and then the second series of measurements was similarly made, as indicated in Figs. 1 and 2. It is well known that a solution having plastic flow may change its structural properties considerably on being forced through a capillary. Hence, *the final measurements in Figs. 1 and 2, made after the appearance of "elasticity," do not really represent 3½ hours of ageing of* the sol but are the measurements after 3½ hours of ageing modified by the disturbance of sol and gel structure produced by the previous measurements.

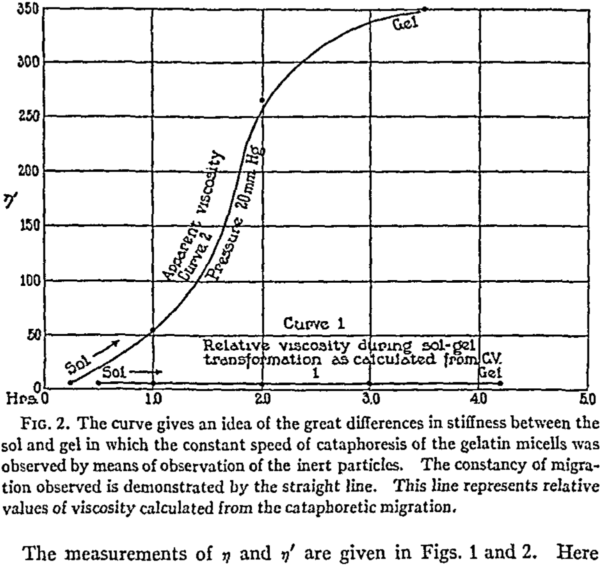

FIG. 2. The curve gives an idea of the great differences in stiffness between the sol and gel in which the constant speed of cataphoresis of the gelatin micells was observed by means of observation of the inert particles. The constancy of migration observed is demonstrated by the straight line. *This line represents relative values of viscosity calculated from the cataphoretic migration.*

The measurements of η and η' are given in Figs. 1 and 2. Here the true viscosity, η, is shown by the initial (15 min.) measurement, and by the measurements at 400 mm. Hg. For the purposes of this communication the initial value of η determined at different pressures was practically independent of the pressure. This initial value of η for different pressures falls, therefore, practically speaking, on the same point. The apparent viscosity, η', varies with the pressure used (as explained above).

From equation (1) and experiment it follows that

$$V = K\frac{1}{\eta} \tag{3}$$

for simple, crystalloidal systems (the symbols as hitherto). From these measurements it may be concluded that *the cataphoretic velocity of gelatin micells (or of particles which are surrounded by a sheath of the gel, and which exhibit an equal velocity) is independent of η', the apparent viscosity of the medium, when η' is of the order of magnitude here.* That is, the Helmhotz-Lamb equation is valid, under the above conditions, for systems such as these gelatin sols and soft gels which have plastic flow. Further, the speed of migration even for small values of H (1 volt per cm.) was proportional to H. It is also to be determined from Fig. 1 that with the ageing of the gelatin sol, and with the appearance of "elasticity," η remains practically unchanged (that is, η' actually approaches η asymptotically with increasing shearing stress). Expressed algebraically, it would appear that the following relationship is probably present:

$$\eta' = f(F) + \eta \tag{4}$$

where F is the shearing stress and η is a constant independent of time of ageing; or, under the above conditions

$$\frac{d\eta}{dt} = 0 \tag{5}$$

where t is the time.[2] This constancy of η has been observed for a variety of other "elastic" sols and gels and is discussed elsewhere (8).

To summarize: from the study of these inert particles, the cataphoretic velocity of a microscopic particle in certain soft gelatin gels is the same as the cataphoretic velocity of the gel itself. This movement is proportional to the applied difference of potential and inversely proportional to the true viscosity of the medium.[3] There exist, then, conditions under which a leucocyte (if it were to behave

[2] Note that the interval from 0 time to 15 minutes has not been here investigated.

[3] This may also be a value which is a direct function of η, but not differing very greatly from it.

like such inert particles could be transported cataphoretically through the gel, by virtue of a surrounding sheath of the gel itself. That this is true is shown experimentally in the following.

The Cataphoresis of Red Cells, Leucocytes and Quartz Particles in Gelatin Sols and Gels.

The preceding observations concerning the migration of certain particles in gelatin gels *have not shown that cataphoretic migration may take place in a gel independent of the movement of the gel itself.* Such independent migration may be demonstrated as follows.

TABLE II.

The Cataphoresis of Red Cells, Leucocytes and Quartz Particles in a 1.2 per cent Gelatin-Serum Gel.

The measurements given here are relative values for the level of the electrophoresis cell where the speed of the water is negligible. E.M.F. about 15 volts per cm. Note the independent migration of the red cells in sol and gel.

Nature of medium	Relative velocity			Remarks
	Red cells	Leucocyte	Quartz	
	μ per sec.	μ per sec.	μ per sec.	
Fresh sol (slightly) "elastic")	12	6	7	The difference between quartz particles and leucocytes are actually less when more accurate determinations are made
40 min. later (soft gel)	11	5	6	The slight decrease in velocity is due to the fact that these values are means of several measurements where the first speeds were lower due to the presence of a slightly stiffer gel. This is demonstrated in Table III where the characteristic cataphoretic thixotropic, *i.e.* softening, is brought about in the gel without completely destroying the gel

If one prepares a 1.2 per cent gelatin sol with horse serum having suspended in it quartz particles (1.0 to 3.0μ in diameter), red cells and leucocytes, the following events take place. As previously mentioned, a freshly prepared sol shows practically no "elasticity." The quartz particles show a distinct Brownian movement and the leucocytes and red cells settle rapidly to the floor of the electrophoresis

cell. Table II gives the relative speeds of these three types of particles for the sol. It is to be noted that the quartz particles move with about the same speed as the leucocytes, but that *the red cells wander approximately twice as fast.*[4] It may be recalled that in serum and in plasma red cells also wander about twice as fast as leucocytes. Whether or not the lipoid surface of the red cell is the cause of this difference is a problem for future discussion. The values previously reported are about 1.0μ per sec. per volt per cm. for red cells and about 0.5μ per sec. per volt per cm. for leucocytes in both media. That is, the leucocytes behave in the gelatin serum medium remarkably as though they had a surface similar to quartz in its response to their common medium. The red cells, however, are distinguished by having a higher velocity.

In the presence of the serum proteins gelatin takes place rapidly, and after a half hour a soft gel has formed. That a gel is present in the electrophoresis cell is proven by the following.

1. A specimen of the sol in a test-tube has stiffened.
2. The blood cells remain suspended at the same level in the cell. It has previously been pointed out that they settle out rapidly in the sol.
3. The quartz particles show no Brownian movement.
4. If the material be sucked out of the cataphoresis cell, it is found to be a soft gel which corresponds to a stiffer gel before the disturbance of extraction.

We may now with certainty consider the migration of the particles in this system as migration in a gel. *The migration of the red cells in such a gel is practically the same as migration in the sol and the same excess of speed is present* (Table II). The red cells here have a mean velocity from the first few measurements of 11μ per sec., about twice that of the quartz and leucocytes. In other words, *the red cell can wander cataphoretically through a soft gel of this type as if relatively no hindering gel structure were present.* The red cells must either push aside or destroy the gel structure during migration.

The experiment may be made still more striking by contrast with a stiffer gel. In a 1½ per cent gelatin serum gel, about 3 hours after the

[4] In serum, quartz particles and leucocytes move with the same cataphoretic velocity. The red cells, of course, have a velocity about twice as great.

sol had been prepared, the process of gelation had formed a stiff gel. In this gel all three types of particles moved at first slowly but with the same speed (Table III). The speed of both quartz particles and red cells increased in successive measurements, but both at about the same rate. Then suddenly the red cells increased their speed about 100 per cent and again assumed their former ability to wander in-

TABLE III.

1½ per cent gelatin serum. This table shows that after softening of the gel (thixotropic effect) by cataphoresis, red cells may wander through relatively concentrated gels as if no gel were present. Numbers before speeds refer to consecutive measurements showing change in velocity with change in gel consistency. This change does not take place in more dilute gels, *e.g.* like those described in Figs. 1 and 2. Note that although a stiff gel is present in the second row, the primary speed is ¼ of that found for red cells in the sol. The increase in η', the apparent viscosity, would be at least 200 × the initial value when measured with a low shearing stress. The criteria of the continued presence of the gel are described fully in the text. Measurements were made in the middle of the cell and are therefore relative.

Nature of medium	Relative speed		Remarks
	Red cells	Quartz	
	μ *per sec.*	μ *per sec.*	
Fresh sol	17	8.5	
Gel (4 hrs.) Successive measurements	(1) 4.5 (2) 5.3 (3) 5.9 (4) 11.4 (5) 13.2 (6) 13.2 (7) 15.5	(1) 4.5 (2) 4.9 (3) 5.1 (4) 5.4 (5) 8.0 (6) 8.3 (7) 8.0	The speed is at first the same. With softening, the distinctive velocity of the red cell (4) manifests itself although a fairly stiff gel is present

dependently through the gel. This did not seem to be due to a heating effect. Nor was the gel made into a sol, for the particles all remained suspended in their original positions. The experiment could be repeated later after restiffening of the gel. The explanation offered is briefly the following. Gelatin has been found to be thixotropic (8). That is, under certain conditions, it may undergo a reversible gel-sol partial transformation by shaking or other mechanical

means. Here, the cataphoretic movement of the micells of the gel past one another (1, 2) partially destroyed the initial rigidity of the gel, but the gel was still so stiff that the particles suspended remained at the same level and showed no Brownian movement. It may be mentioned, however, that the study of quartz particles in a 1.0 per cent pure gelatin gel like that described by the curves of Fig. 1 showed no change in speed or softening with successive measurements. The gel in this case was so soft that *cataphoretic* movements of the micells were not hindered by the gel structure. The partial destruction of the gel structure takes place without influencing the migration of particle as the true viscosity is the function here determining speed of cataphoresis.

The foregoing experiments prove conclusively that microscopic particles may wander cataphoretically through certain gelatin gels with a velocity which, is within limits, practically independent of the structure of the gel.[5] This independent migration is also manifested in soft gels of much higher protein concentration. That similar mechanism could be employed by a leucocyte wandering through the gel structure of the capillary wall is self-evident. This question will subsequently be discussed in detail.

The Cataphoresis of Leucocytes in Dilute Fibrin Gels.

A clear difference in the behavior of leucocytes wandering cataphoretically in gelatin and dilute fibrin gels can be demonstrated. Whereas in gelatin gels leucocytes and red cells migrate at a given level in the cell with their respective velocities, the migration in the fibrin gel is totally different. The strands of fibrin offer a *mechanical* solid resistance. A cell moving rapidly may suddenly come to a complete stop as it hits a strong fibrin strand. Thinner strands may break temporarily hindering migration. Or there may be places where there is no hinderance to migration. A solid strand may then suddenly prevent further movements of the particle which may or may not eventually edge its way past. This is a noteworthy difference

[5] This is somewhat similar to the findings of Laing (*J. Phys. Chem.*, 1924, xxviii, 673 for Na oleate sols and gels), who investigated submicroscopic particles. Arrhenius in 1887 reported that gel structure did not hinder ion transport.

in structure between the gelatin and fibrin gel brought out by electrophoresis of large particles. Silica gels, soap gels, iron oxide and other gels remain for future similar investigations.

The Influence of an "Elastic" Medium on the Migration of Particles When the Force Is Not a Difference of Potential.

Let it be assumed that the leucocytes are made to wander in an "elastic" gel by a force similar to surface tension or by some other force which does not influence the medium. In this case, the movements would be similar to the movements of nickel particles in a gel when the paticles are made to move by a unipolar electromagnet. The observations of Freundlich and Seifriz (9) have been in part confirmed by the author and in general may be described as follows.

A nickel particle suspended in a viscous medium like glycerol moves when a slight magnetic force is applied. When the magnetic force is stopped, the particle remains in the place where it was at the cessation of the flow of the current.[6] An entirely different type of movement occurs in an "elastic" medium like gelatin. Upon application of a very slight magnetic force, no easily perceptible movement takes place. When the force is increased slightly, the particle starts to move and then stops, even though the magnetic force is still present. If the current in such a case be now shut off, the particle returns to its original place in the "elastic" sol or gel.[7] It is only with magnetic forces of greater magnitudes, with partial destruction of the gel structure, that a progressive movement of the particle toward the magnet takes place. Comparing, then, the ease of movement of a particle through a gel by means of such a force and the cataphoretic movement, a particle would seem to suffer much less resistance to migration in the "elastic" medium if differences in potential were responsible for the migration. The shear between the particle and the medium when the movement is cataphoresis must produce sufficient destruction of the soft (*e.g.* 1 per cent) gelatin gel to allow migration to take place unhindered by the remaining rigid but partially destroyed gel structure.

DISCUSSION.

According to Krogh (10) the normal thickness of capillary endothelium is less than 1.0μ. Considering that a leucocyte wanders through the thinnest part of the capillary wall, it is compatible with

[6] The "current" here refers to that flowing in the magnet.

[7] Such a "jumping back" phenomenon may be observed in old or stiff gels at the end of cataphoresis measurements.

our knowledge of leucocyte emigration to take as the probable limits of capillary wall thickness, where emigration occurs, 0.1 to 0.5μ. It has been shown in the first paper of this series (1) that injured connective tissue is most probably electropositive to the blood stream, with the negative current therefore flowing toward the point of injury. Using the same mode of reasoning it follows that the side of the capillary wall in contact with the injured connective tissue should be positive relative to the blood stream, although negative to the zone of injury. Table IV shows the calculated drop in potential per cm. for thicknesses of capillary wall from 0.1 to 0.5μ, calculated for membrane potentials from 0.1 to 10.0 millivolts. These values are from 2 to 1000 volts per cm. It may be recalled that a leucocyte

TABLE IV.

This table demonstrates that with even exceedingly slight membrane potentials the order of magnitude of the potential drop per cm. should be sufficient to produce cataphoretically leucocyte emigration through the capillary wall.

Thickness of capillary wall	0.1 μ	0.5 μ
Possible membrane potentials across capillary wall	Calculated drop in potential across membrane (capillary wall)	
volts.	*volts per cm.*	*volts per cm.*
.0001	10.0	2.0
.001	100.0	20.0
.01	1000.0	200.0

wanders in serum and plasma with a cataphoretic velocity of about 0.5μ per sec. per volt per cm. (1, 2). It is at once evident from the table that even in the most extreme case a sufficient difference of potential should be present to cause particle emigration with an appreciable speed (Fig. 3). As the leucocyte wanders through the gel the random ameboid movements should aid in destroying the gel structure. That is, a thixotropic effect, a loss in rigidity of the gel, should occur incidental to the mechanical disturbances present. Migration could occur in two ways. The migration of the cell could take place as it does in the gelatin gel, *i.e.*, moving with the same speed as the proteins of the gel in the electrical field as if it were surrounded by a sheath of the protein composing the medium. Or, migration

could take place *independently* of the presence of the gel, similar to the independent cataphoretic movement of the red cells in the gelatin gels.[8] The directing force of a difference of potential is also evident in the event that the cell meets an obstruction like the strands of a fibrin gel.

SUMMARY.

1. Quartz particles and certain other particles move cataphoretically in certain soft gelatin gels, with the same velocity as in the sol. The speed is a function of the true viscosity of the sol or gel, and it is

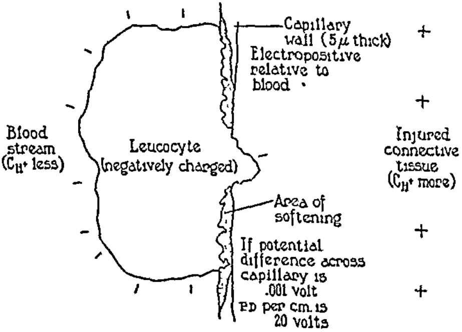

FIG. 3. Schema to show proposed mechanism of leucocyte emigration. For further details the text should be consulted.

apparently not altered in these soft gels by the presence of gel structure. It is proportional to the applied difference of potential.

2. This finding is compatible with the fact that certain sols undergo gelation with no increase of the true viscosity although a marked change in the apparent viscosity takes place.

3. Red cells in soft gelatin-serum gels show a distinct difference in behavior. They migrate through the sol or gel with a speed that is about twice as great as the leucocytes and quartz particles, which

[8] In view of the data concerning the surface of the leucocyte, the former process is the more likely.

latter particles migrate with the same velocity. This ratio has been found to hold for serum and plasma. The absolute velocities are comparatively slightly decreased by the presence of the gel.

4. In more concentrated or stiffer gels, leucocytes, red cells and quartz particles all move at first with the same velocity. By producing mechanical softening of these gels (shearing from cataphoretic movement of the micells within the cell) the red cells presently resume their previous property of independent migration through the gel.

5. The movements of particles in gelatin gels produced by a magnetic force or the force of gravity are of a different nature than those movements produced by cataphoresis.

6. The mechanical nature of obstruction to the cataphoretic migration of leucocytes and red cells in fibrin gels is briefly described.

7. The correlation of cataphoresis of microscopic particles in gels with the order of magnitude and nature of the potential differences in the capillary wall, lends additional evidence to the theory that polymorphonuclear leucocyte emigration and migration are dependent upon these potential differences.

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THE INFLUENCE OF SURFACE CHARGE AND OF CYTOPLASMIC VISCOSITY ON THE PHAGOCYTOSIS OF A PARTICLE

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Of the several physical factors which may influence phagocytosis, the effect of surface charge on cell and particle and the effect of cytoplasmic viscosity have not received such extensive attention as have such factors as accidental contact of cell and particle, "surface stickiness," and surface tension conditions at cell and particle interfaces. Fenn has referred to both of these factors in the course of a consideration of other aspects of the problem (1, 2), and Leo Loeb has emphasized the importance of cytoplasmic viscosity (3), but we are unable to find more complete references to the subject.

The object of this paper is to consider in some detail the influence of surface charge and of cytoplasmic viscosity on the act of ingestion. As will be seen below, it is impossible to do this in other than a very general fashion, partly because the method of treatment necessarily rests on uncertain assumptions, and partly because a number of unknown and apparently undeterminable constants make their appearance as the treatment proceeds. Unsatisfactory as this may be, the methods used are sufficient to define the nature of the variables involved, and for this reason the results may have a certain interest as approximations.

1. Surface Charge.

The effect of a like surface charge on cell and particle may be regarded from two points of view. In the first place, the charge may influence the *approach* of the particle to the cell by making the approach more difficult or easier according as to whether the charge gives rise to a repulsion or an attraction between the approaching

bodies. Usually the presence of a like charge results in a repulsion, and would always do so were no redistribution of charge possible; we have, however, already suggested that this usual repulsion may be replaced under certain circumstances by an attraction, due to a reversal of sign of the charge on the surface of the particle (4). Secondly, the surface charge may affect the ingestion of the particle *once contact has occurred*, for then the ingestion is determined by the ratio $(S_1 - S_{12})/S_2$, where S_1, S_{12}, and S_2 are interfacial tensions which will be modified by surface charges if such exist. In a sense, although they both depend fundamentally on the same conditions, these two effects of surface charge are distinct; the first effect manifests itself when the cell and particle are a small but appreciable distance apart, but the second can obviously refer only to a condition in which the interfacial tension S_{12} exists, *i.e.*, where cell and particle are in contact.

In what follows we propose to ignore altogether the possible effect of surface charge on the approach of cell and particle and to consider its effect on ingestion alone, just as in a previous paper we considered the effect on approach alone without taking into account the influence which the charge might have after contact between cell and particle had been established. We also make the assumption that redistribution of charge does not occur, whereas we previously assumed, in dealing with the other aspect of the problem, that it does.* This piecemeal treatment is necessary in view of the complexity of the problem, and must be clearly recognized to be limited by the incompleteness of the initial assumptions.

Consider a system of N large spheres (cells) or radius R, and of n little spheres (particles) of radius r. Let the tension and charge at the interfaces between the large spheres and the suspending fluid be σ_0 and ω. Suppose that n, the number of little spheres, can be

* In dealing with this other aspect of the problem, we have treated the charges upon cell and particle as electrostatic charges, although it is the case that no single suspended cell or particle has a free charge. The justification for this procedure is that the repulsive effects manifested between similarly charged particles or between cells and particles must be associated with open tubes of force. During the time when these repulsions occur, the charges may probably be treated as free without the introduction of much error. We admit, however, that this is a controversial point.

divided up into n_1, the number ingested by or contained within the large spheres, and n_2, the number left uningested. At the surfaces of the group n_1 let there be tension σ_1 and charge $k_1\,\omega$, and at the surfaces of the group n_2 let there be tension σ_2 and charge $k_2\,\omega$. Let d denote the thickness of the double layer at every interface. The problem is to find the value of ω which will prevent the ingestion of the little spheres by the large ones, given initially that r is small compared to R.

To do this, let us consider the conditions which will put the energy of the whole system at a minimum. The assumptions upon which we shall proceed are those made by Gyemandt in dealing with the simple case of agglutination of two similar particles; Gyemandt has discussed the validity of these assumptions in full, and we refer the reader to his monograph (5).

If r is small compared with R, we may neglect the increase in the radius of the large spheres when the little ones are ingested. The total energy of the whole system is given by the expression

$$E = 4\,\pi\,R^2 N\left(\sigma_0 + \frac{\lambda\,d\,\omega^2 R}{R+d}\right) + 4\,\pi\,r^2 n_1\left(\sigma_1 + \frac{\lambda\,dk_1^2\,\omega^2 r}{r+d}\right) + 4\,\pi\,r^2 n_2\left(\sigma_2 + \frac{\lambda\,dk_2^2\,\omega^2 r}{r+d}\right) \quad (1)$$

where

$$\lambda = 2\,\pi/e,$$

e being the dielectric constant.

Vary $R_1\ \omega_1\ n_1$ and ω_2. Since $-\delta n_1 = \delta n_2$, the total energy of the whole system is at a minimum when

$$\left\{2\,R\,N\,\sigma_0 + \frac{\lambda\,d\,\omega^2\cdot N\cdot 3\,R^2}{R+d} - \frac{\lambda\,d\,\omega^2\,N\,R^3}{(R+d)^2}\right\}\delta\,R$$

$$+\left\{\frac{R^3\,N\cdot\lambda\,d\cdot 2\,\omega}{R+d} + \frac{r^3\cdot\lambda\,d\,2\,\omega\,(n_1\,k_1^2 + n_2\,k_2^2)}{r+d}\right\}\delta\,\omega$$

$$+\,r^2\left\{\sigma_1 - \sigma_2 + \frac{\lambda\,d\,\omega^2\,r\,(k_1^2 - k_2^2)}{r+d}\right\}\delta\,n_1 = 0 \quad (2)$$

or

$$A_1\,\delta R + B_1\,\delta\omega + C_1\,\delta n_1 = 0$$

Introducing the assumption that the total charge remains constant,

$$2\,\omega \cdot RN \cdot \delta R + (R^2N + k_1\,n_1\,r^2 + k_2\,n_2\,r^2)\,\delta\omega + \omega r^2\,(k_1 - k_2)\,\delta n_1 = 0 \tag{3}$$

or

$$A_2\,\delta R + B_2\,\delta\omega + C_2\,\delta n_1 = 0$$

Further, the volume of the system must remain constant, and so

$$\delta R = \frac{-\,r^3}{3\,N\,R^2}\,\delta n_1 \tag{4}$$

Thus we have

$$r^3\,(A_1\,B_2 - A_2\,B_1) = 3\,NR^2\,(C_2\,B_1 - C_1\,B_2)$$

We now substitute the values of A_1, A_2, B_1, B_2, C_1, and, C_2 and, after a very long process of simplification, obtain

$$\lambda\,d\omega^2 = \frac{\begin{array}{c} 3\,R\sigma_2\,\{NR^2 + r^2\,(k_1\,n_1 + k_2\,n_2)\} \\ -\,3\,R\sigma_1\,\{NR^2 + r^2\,(k_1\,n_1 + k_2\,n_2)\} \\ -\,2\,\sigma_0\,\{R^2rN + r^3\,(k_1\,n_1 + k_2\,n_2)\} \end{array}}{\begin{array}{c} 3\,Rr\left\{\dfrac{NR^2 + r^2\,(k_1\,n_1 + k_2\,n_2)}{R + d}\right\} - R^2r\left\{\dfrac{R^2N + r^2\,(k_1\,n_1 + k_2\,n_2)}{(R + d)^2}\right\} \\ +\,\{6\,R\,(k_2 - k_1) - 4\,r\}\left\{\dfrac{NR^3}{R + d} + \dfrac{r^3\,(n_1\,k_1 + n_2\,k_2^2)}{r + d}\right\} \\ -\,3\,Rr\,(k^2 - k_1^2)\left\{\dfrac{NR^2 + r^2\,(k_1\,n_1 + k_2\,n_2)}{r + d}\right\} \end{array}} \tag{5}$$

as the critical value of ω which, if exceeded, will prevent ingestion of the little spheres occurring. This expression verifies for Gyemandts' case of two similar particles.

Put

$$(k_1\,n_1 + k_2\,n_2) = \varphi \text{ and } (NR^2 + \varphi r^2) = \theta;$$

Then, approximately,

$$\lambda\, d\omega^2 = \frac{(3\, R\sigma_2 - 3\, R\sigma_1 - 2\, r\sigma_0)\, \theta}{3\, r\theta - r\theta - 3\, R\theta\, (k_2^2 - k_1^2) + \{6\, R\, (k_2 - k_1) - 4\, r\}\, \theta}. \tag{6}$$

θ cancels out; the critical charge is therefore independent of N, n_1, and n_2, as it ought to be. If we now neglect r as compared with R, we get

$$\omega = \sqrt{\frac{3\, R\sigma_2 - 3\, R\sigma_1 - 2\, r\sigma_0}{\lambda\, d\, \{2\, r - 3\, R\, (k_2 + k_1 - 2)\, (k_2 - k_1)\}}} \tag{7}$$

as the critical charge.

For ω to be real, ω^2 must be positive, and the numerator must have the same sign as the denominator. This occurs only under certain conditions, and, as there are so many factors which may determine the sign of numerator and denominator, these conditions are exceedingly complex. Three, however, are sufficiently important to set down.

(*a*) If σ_1 (the tension at the cell-particle interface) is greater than σ_2 (the tension at the fluid-particle interface) the numerator must always be negative. A real value of ω will result only if the denominator is negative also, *i.e.*, if k_2 is sufficiently greater than k_1. Thus only a large surface charge at the fluid-particle interface can influence the ingestion.

(*b*) If σ_2 is greater than σ_1 the numerator may be positive or negative according to the value of σ_0. If σ_0 is small and if k_1 is sufficiently greater than k_2, ingestion will be prevented, for ω will be real; a large surface charge at the cell-particle interface may thus influence the ingestion process. If, however, σ_0 is large, the numerator will be negative, and ingestion will be prevented only if k_2 is sufficiently greater than k_1. A special case of this is the case considered by Gyemandt, where $\sigma_1 = k_1 = 0$, and where there is a real value of ω.

(*c*) The critical charge ω is a function of both R and r, or rather of the ratio R/r. The way in which the critical charge varies with the size of cell and particle respectively is, however, a very complex matter, for it depends on the values of σ_0, σ_1, and σ_2, as well as on the values of k_1 and k_2. If, for instance, we fix R and vary r, in some

cases we find the critical charge increasing as r increases, and in other cases we find it diminishing as r increases. This makes it possible, so far as this treatment is concerned, for a large particle to be more readily ingested than a small one, or *vice versa*, depending on the conditions in the system. In the case which corresponds to that dealt with by Gyemandt, however, the same result as he records is obtained, for an increase in the value of r gives a decrease in the critical charge.

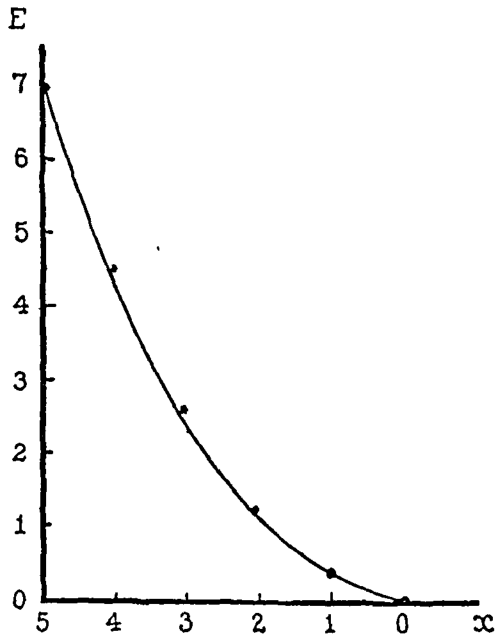

FIG. 1. Graph showing values of E, the total energy of the whole system in excess of the energy $E = 0$ when $x = 0$. Black dots indicate values calculated from $E = k \cdot x^2$ (Graph from Fenn's data).

In concluding this part of the investigation, it must be admitted that the results are very unsatisfactory. Although the approximate effect of surface charge and surface tension on ingestion is defined, the definition is of little use for practical purposes, for it contains too many undeterminable constants.

2. Cytoplasmic Viscosity.

The study of the effect of cytoplasmic viscosity on ingestion of a particle is much more interesting than is the study of the effect of

surface charge, not because the results are any more useful, but because they are rather surprising.

Let us first consider the entry of a rigid spherical particle into a spherical cell of viscosity η. The velocity of movement of the particle into the cytoplasm will depend on (1) the magnitude of the forces pulling it in, and (2) the magnitude of the resistance which it encounters to its motion. In order to define these factors further, let us restrict ourselves to the consideration of a case in which equilibrium is reached at complete ingestion only ($\cos \theta > +1$). Call the radius of the cell R, and the radius of the particle r; further, let R be great as compared with r. Suppose the particle moves into the cell along an axis x in the direction of x-negative, and that equilibrium takes place when the centre of the particle reaches the point $x = 0$; then when the particle is at the cell surface $x = 2r$, when it is half inside $x = r$, and when ingestion is complete $x = 0$.

(*a*) If we can find E, the energy of the whole system in excess of a value $E = 0$ when $x = 0$, and can find it as a function of x, the force pulling the particle inwards will be dE/dx, for the direction of motion is along the x-coordinate only. Unfortunately, however, it is impossible to find E as a function of x by geometrical procedures, and so it is necessary for us to find a graphical relation for different arbitrary values of R and r. Such a relation has been worked out by Fenn, and is figured in one of his papers (6); this relation between E and x, expressed in arbitrary units, supplies us with all we require. The curve is well expressed by $E = kx^2$ (k in this case is 2.77), and the extent to which this empirical formula fits can be judged by an inspection of Fig. 1. The fit is remarkably satisfactory.

The force pulling the particle in is also, however, a function of r, the radius of the particle, and becomes greater as the particle becomes greater. This fact must be introduced into the empirical formula given above, for the curve shown in Fig. 1 is constructed for a special case where $R = 4r$. Since the dimensions of E are those of r^2, while the dimensions of x are those of r, we may write the force pulling the particle in as

$$F_1 = k_1 \cdot r \cdot x \qquad (2)$$

as a first, but quite good, approximation.

(*b*) The resistance which the entering particle experiences to its motion constitutes a more difficult problem. If we can neglect the portion of the particle which is in contact with the fluid as experiencing very little resistance and consider only the part inside the cell as being subject to appreciable retarding forces, the particle may be regarded as a spherical segment of varying height moving through the fluid cytoplasm with its base in a plane at right angles to the direction of motion x. Provided that we consider only the movement of the particle between the limits $x = 2r$ and $x = r$, so that we restrict ourselves to the first half of the ingestion process, we can arrive at an approximation to the resistance offered by the method of dimensions.

Let the force F_2 opposing the motion of the particle between the mits $x = 2r$ and $x = r$ be

$$F_2 = k_2 \cdot \rho^v \cdot d^x \cdot \eta^y \cdot V^z$$

where ρ is the radius of the base of the spherical segment referred to, η the viscosity of the cytoplasm of the ingesting cell, d its density, V the velocity of motion of the particle, and k_2 a dimensionless constant.

For equal dimensions in M,

$$x + y = 1$$

For equal dimensions in L and T,

$$v - 3x - y + z = 1$$
$$- y - z = -2$$

From these equations,

$$F_2 = k_2 \left(\frac{V \rho d}{\eta}\right)^z \cdot \frac{\eta^2}{d}.$$

If V is small, as it will be in this case, z is generally unity, and so we have the result that

$$F_2 = k_2 \eta \rho V \qquad (9)$$

just as in the case of the motion of a sphere, except that k is not equal to 6π and that ρ is a function of x instead of a constant. The resistance to the motion of the particle will thus increase as the particle

moves along the axis of x from $x = 2r$ to $x = r$, for ρ equals $\sqrt{r^2 - (x-r)^2}$, or $\sqrt{2rx - x^2}$, and increases from 0 to r between these limits.

Now if the viscosity of the cell is so great that the motion of the particle is slow, the particle may be considered as taking up a series of "terminal velocities" given by

$$F_1 = F_2.$$

From (8) and (9), this is equal to

$$\frac{dx}{dt} = \frac{k_1 r \cdot x}{k_2 \rho \cdot \eta}$$

whence

$$t = \frac{k_2 \eta}{k_1 r} \int \frac{\sqrt{2rx - x^2} \cdot dx}{x}$$

provided that the value of the integral is not taken outside the limits $x = 2r$ and $x = r$. Remembering that the movement of x is measured in the direction of x-negative, and that when $t = 0$, $x = 2r$, we obtain from this the expression

$$t = \frac{k_2 \eta}{k_1 r} \left\{ r \cdot \sin^{-1} \sqrt{1 - \frac{r^2 - 2rx - x^2}{r^2}} - \sqrt{2rx - x^2} \right\} \tag{10}$$

The rate of ingestion is thus first rapid and then slower, and the time taken for half ingestion varies directly with the cytoplasmic viscosity and inversely as the radius of the particle.

It is interesting at this point to stop to enquire what effect cytoplasmic viscosity would have on ingestion if the cell and particle were at rest in a stationary fluid. Any particle which came into accidental contact with the cell would then be ingested (surface tension conditions, etc., being supposed to be favourable) and the rate of ingestion would depend on the viscosity of the cytoplasm. Even if the cytoplasmic viscosity were very great, ingestion would ultimately occur, although with exceeding slowness, for there would be no forces which would tend to remove the particle from the surface of the cell or which would oppose the forces drawing the particle inwards.

If, however, the cell is moving through a viscous fluid such as a suspension medium, or if, what amounts to the same thing, a viscous fluid is moving steadily over the cell, forces arise which tend to remove the particle from the cell surface and which thus tend to

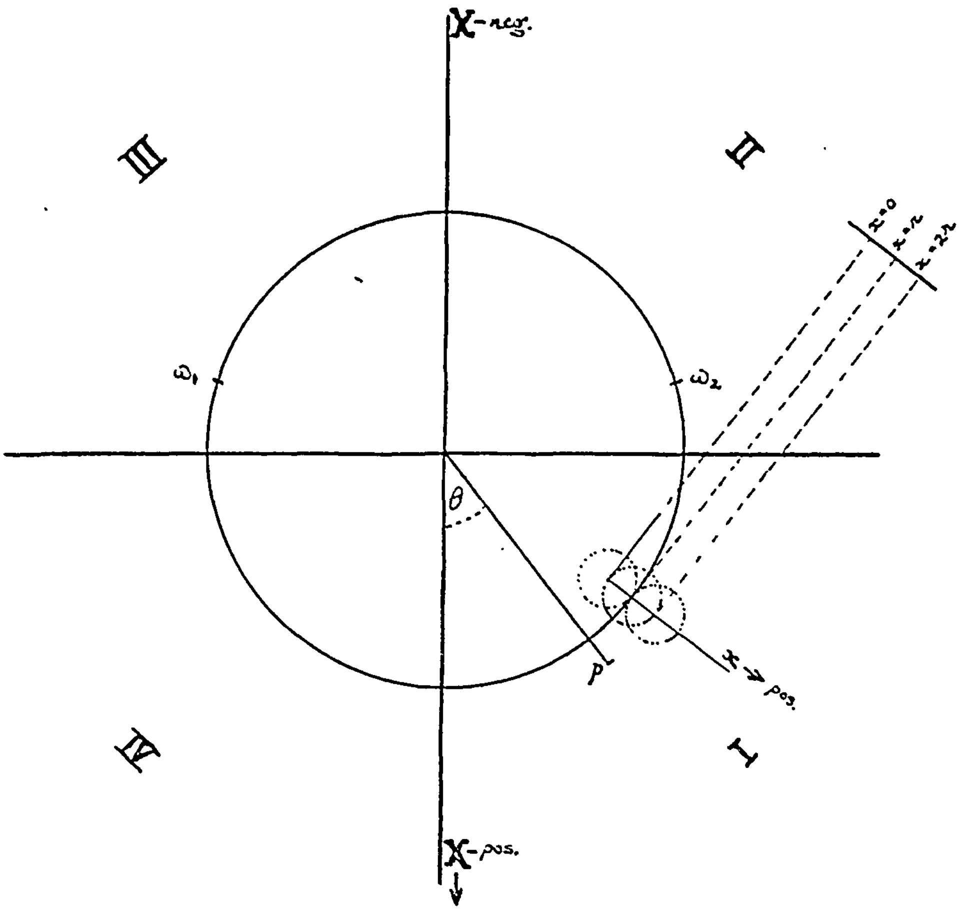

FIG. 2.

prevent its ultimate ingestion. The magnitude of these forces has now to be considered.

In Fig. 2 the cell of radius R is moving in a viscous fluid with a velocity U along an axis of X in the direction X-positive. Suppose that a small particle is situated at the cell surface in a position p, θ, with respect to the centre of the cell as origin of the polar coordinates.

In the absence of the particle, the velocity-potential at p, θ, would be

$$\psi = \frac{3UR \cdot p}{4} \cdot \left(1 - \frac{R^2}{3p^2}\right) \cdot \sin^2 \theta, \tag{11}$$

and this we shall take as the approximate value of the velocity-potential at p, θ, even if the particle is situated at that point.

The component velocities along and at right angles to the radius vector are

$$\lambda = \frac{1}{-R \cdot \sin \theta} \cdot \frac{\delta\psi}{R\, \delta\theta}$$

and

$$\mu = \frac{1}{R \cdot \sin \theta} \cdot \frac{\delta\psi}{\delta R}$$

or

$$\lambda = U \cdot \cos \theta \left(1 - \frac{R^3}{2p^3} - \frac{3R}{2p}\right), \tag{12}$$

and

$$\mu = -U \sin \theta \left(1 + \frac{R^3}{4p^3} - \frac{3R}{4p}\right). \tag{13}$$

In interpreting these component velocities attention must be paid to the sign of $\cos \theta$ and to the sign of the component velocity λ or μ. To facilitate this, Fig. 2 has been divided up into quadrants symmetrically placed about the axis of X. In quadrant I, $\cos \theta$ varies from 1 to 0, and λ is negative near the cell surface; in quadrant II $\cos \theta$ varies from 0 to -1, and λ is positive instead of negative.

These component velocities will, of course, give rise to forces which will not be without their effect on the particle situated at the surface of the cell. The component λ, acting along the radius vector, along

which, it will be remembered, the forces F_1 and F_2 also act, will tend to aid the passage of the particle into the cell or to prevent its passage according to the sign which the component possesses, or, to put it another way, the force dependent on λ will either increase or diminish the magnitude of F_1. The component velocity μ will always give rise to forces which act at right angles to the radius vector, and will therefore be without effect on the forces F_1 and F_2; the forces which result from this component will have the effect of producing a rotation of the cell plus particle or of moving the particle through an angle to a new part of the cell surface, and therefore we can ignore their effect in the meantime.

The consideration of the velocity component λ on the motion of the particle provides quite enough difficulty. Because of its presence, a force F_λ will be generated, and the particle will thus be acted upon by two forces, F_1 and F_λ, which may either act in the same direction or oppose one another according to the sign of the latter. If F_λ is negative, as in quadrant I, they will act together in the same direction; if F_λ is positive, as in quadrant II, they will act against each other. The total force acting along the radius vector and tending to move the particle along the axis of x will thus be $F_1 - F_\lambda$. If F_λ is negative or is positive and less than F_1, the particle must move in the direction x-negative, *i.e.*, into the cell; if, on the other hand, F_λ is positive and greater than F_1, the particle will be moved in the direction x-positive, and will tend to be withdrawn.

We have now to define the force F_λ in terms of the proper variables. The force generated by a moving stream of a certain velocity is proportional to the velocity and to the projected surface area of the body upon which the force acts, and so we can take F_λ to be

$$k_3 \cdot U \cdot \cos\theta \cdot \left(1 - \frac{R^3}{2p^3} - \frac{3R}{2p}\right) \cdot \pi r^2 \tag{14}$$

when the particle is moving in the direction x-negative, and approximately equal to the similar expression

$$k_4 \cdot U \cdot \cos\theta \cdot \left(1 - \frac{R^3}{2p^3} - \frac{3R}{2p}\right) \cdot (\pi r^2 - \pi\rho^2) \tag{15}$$

when the movement is in the direction x-positive, the expressions being taken, as before, as applying to the motion between $x = 2r$ and $x = r$ only. Both expressions are, of course, very approximate only, for they assume that the presence of the particle does not materially alter the form of the stream-lines at the cell surface. The approximations are, however, sufficiently good to lead us to the general conclusion.

The difficulty now arises that F_λ is a function of R and p instead of a function of x and r, and so it is necessary to transform it. To do so, we take the part of the expressions which contain R and p, and, putting $R = 1$, plot the value of

$$\left(1 - \frac{R^3}{2p^3} - \frac{3R}{2p}\right) = \varphi \tag{16}$$

for different values of p from $p = 1.0$ to $p = 1.3$. This takes us sufficiently far from the cell surface for the result to cover all cases in which R is great compared to r. The result is rather surprising, for it appears that φ is very nearly a linear function of p. This is shown in the following table of values, in which the calculated values of φ, obtained from the linear expression given below, are inserted for comparison with the real values.

p	φ	φ, calculated
1.3	−0.38	−0.34
1.2	−0.54	−0.55
1.1	−0.74	−0.77
1.05	−0.87	−0.88
1.0	−1.00	−1.00

From these figures it appears that we may use as a very fair approximation indeed the relation

$$\varphi = (2.22p - 3.22) \tag{17}$$

in place of the part of the expression which gives F_λ in terms of R and p. This simplifies matters considerably, as will be seen below.

The simplification arises from the fact that p is expressible in terms

of x and r. To take one case where $r = 0.1R$, and where R is equal to unity,

when

$$p = 1.0,\ x = r$$
$$p = 1.1,\ x = 2r,$$
$$p = 1.2,\ x = 3r,$$

and so on, or, in general,

$$x = R(p - 1) + r,$$

and

$$p = \frac{x - r}{R} + 1.$$

Thus we have

$$\lambda = U \cdot \cos\theta \cdot \left\{2.22\left[\frac{x - r}{R} + 1\right] - 3.22\right\}$$
$$= U \cdot \cos\theta \cdot \left\{\frac{2.22\ (x - r)}{nr} - 1\right\}$$

where $R = nr$. Once more it is necessary to note the limits within which this expression is applicable as an approximation, for when $x = r$, $\lambda = -U \cdot \cos\theta$. Taking now the case in which the particle is moving in the direction x-negative, we have, in place of (14),

$$F_\lambda = k_3 \cdot U \cdot \cos\theta \cdot \left\{\frac{2.22\ (x - r)}{nr} - 1\right\} \cdot \pi r^2 \tag{18}$$

and the velocity of motion

$$\frac{dx}{dt} = \frac{k_1 \cdot r \cdot x - k_3 \cdot U \cdot \cos\theta \cdot \left\{\frac{2.22\ (x - r)}{nr} - 1\right\} \cdot \pi r^2}{k_2 \cdot \eta \cdot \rho} \tag{19}$$

The integration necessary for the finding of the value of t is a very tedious one. The differential equation takes the form

$$t = \frac{A}{E}\int \frac{\sqrt{2rx - x^2}\cdot dx}{x + c}$$

where A, E, and c are complex constants. As the integration is carried out, three terms appear; two of these are large compared with the third term, which is the most complex of all and sufficiently small to be neglected. Much of the complexity arises from the fact that when $t = 0$, $x = 2r$, the direction of motion being in the direction of x-negative. It is not necessary to give more than the final solution, which is

$$t = \frac{k_2 \cdot \eta \cdot}{k_1\, r - B}\left\{(c + r)\cdot \sin^{-1}\sqrt{1 - \frac{r^2 - 2rx + x^2}{r^2}} - \sqrt{2rx - x^2},\right\} \quad (20)$$

where the constant B has the value

$$2.22\ k_4 \cdot U \cdot \cos\theta \cdot \pi r/n \quad (21)$$

and where the constant c has the value

$$\frac{(k_4 \cdot U \cdot \cos\theta \cdot \pi r/n)\ (2.22 + n)r}{k_1\, r - B} \quad (22)$$

Thus t is great when η is great and small when U is great; further, if $U = 0$ or if $\cos\theta = 0$, the whole expression reduces to expression (10), as it should do.

Now suppose that the particle is moving in the direction x-positive, which it will do if F_λ is positive and greater than F_1. In place of expression (14) we now have expression (15), and in place of expression (18) we have

$$F_\lambda = k_4 \cdot U \cdot \cos\theta \cdot \left\{\frac{2.22\ (x - r)}{nr} - 1\right\} \cdot \{\pi r^2 - \pi\rho^2\}. \quad (23)$$

The differential equation corresponding to (19) accordingly is

$$\frac{dx}{dt} = \frac{k_1 \cdot r \cdot x - k_4 \cdot U \cdot \cos\theta \cdot \left\{\frac{2.22\ (x - r)}{nr} - 1\right\}\{\pi r^2 - \pi\rho^2\}}{k_2 \cdot \eta \cdot \rho} \quad (24)$$

This differential equation reduces to the form

$$t = A \cdot \int \frac{\sqrt{2rx - x^2}\, dx}{Bx - (Ex - F)(r - x)^2} \tag{25}$$

which, so far as we can see, is non-integral. A graphical solution between limits would be possible, but is scarcely worth while in view of the complexity of the constants.

We are now in a position to apply this information to the problem of the ingestion of a particle by a cell which is moving through a fluid with a velocity U along a fixed axis X in the direction X-positive. Two cases arise.

(*a*) If F_λ is smaller than F_1, the particle must be drawn into the cell irrespective of the point upon the cell surface at which contact occurs. The time which it will take to pass to a position of half ingestion will be given by expression (20), and will be great if the viscosity of the cell is great. Its magnitude will depend on the excess of F_1 over F_λ in the quadrants where F_λ is positive, and on the sum of the two forces in the quadrants where F_λ is negative. The essential point is that upon whatever part of the surface the contact between cell and particle takes place the ultimate fate of the particle will be complete ingestion.

(*b*) If, at points on the surface, F_λ is greater than F_1, ingestion will occur in quadrant I and the quadrant symmetrical with it, at all points in quadrant II and the quadrant symmetrical with it at which F_1 exceeds F_λ, but not at any point in these latter quadrants at which F_λ exceeds F_1. F_λ cannot, of course, be greater than F_1 at all points in quadrant II, for when $\cos\theta = 0$, $F_\lambda = 0$. Suppose, however, that over a portion s of the entire surface S the excess of F_λ over F_1 exists; the effective surface of the cell for ingestion of a particle will then be reduced from S to $(S - s)$, and the number of particles ingested under ideal circumstances by such a cell as compared with the number ingested by a cell at rest in a fluid will be $(S - s)/S$. This fraction must always be greater than 0.5.

The case of a cell moving steadily along a fixed axis without rotation is scarcely one which could occur in practice, firstly because it is impossible in experiment to prevent irregular motion in the fluid and secondly because the velocity component μ always tends to set

up a rotation of the cell. The case to be considered is therefore that of a system of rotating cells in a fluid, and under these circumstances the conditions for the ingestion of a particle are a little more complex. Statistically speaking, the effect of rotation can be considered as the effect of the uniform rotation of a single cell which is at the same time moving through the fluid with velocity U.

The problem is best approached by considering what will happen to a particle which makes contact with the cell at the point where $\theta = 90°$ between quadrant I and quadrant II, the rotation of the cell being imagined to be clockwise. At the moment of contact, the position of the particle will be such that $x = 2r$, and the force pulling it into the cell will be F_1 only. It will accordingly begin to pass into the cytoplasm according to expression (10), but during its passage the cell will rotate so that the particle will come to lie at successive points on the surface where there is a negative value of $\cos \theta$. The force F_1 will thus be augmented by the force F_λ, and the passage of the particle into the cell will be accelerated according to expression (20), the velocity being at a maximum when the value of $\cos \theta$ is -1, or when rotation has occurred through 90°. As the rotation continues and the particle passes into quadrant IV, the passage into the cell continues according to expression (20) but at a steadily decreasing rate; finally, when rotation through 180° has occurred, the particle lies at the point of junction between quadrants III and IV, and the force acting upon it is again F_1.

Suppose that the rotation through 180° takes place in time t. During this time the particle will have passed into the cytoplasm of the cell for a distance x, dependent on the value of F_1 and on the mean value of F_λ as well as on the viscosity of the cell; the less the viscosity, the greater will be this distance. Two cases now arise. At the end of time t the particle may have passed to the position $x = r$, in which case it cannot be withdrawn, or it may have passed to a position between $x = r$ and $x = 2r$, in which case we have to consider the effect of further rotation. The cell now rotates so that the particle passes into quadrant III. At the moment of entering this quadrant it is acted upon by the force F_1, but as rotation proceeds it is acted upon in the opposite direction by the force F_λ, which is now positive; if a point is reached where F_λ exceeds F_1, the motion of the particle

becomes reversed so that it is withdrawn from the cell, and the velocity of motion will be described by expression (24). Suppose that this withdrawal begins at a point ω_1 and continues, through a maximum velocity at the junction of quadrants III and II, until a point ω_2 is reached; during this period of withdrawal the particle may either be withdrawn to the point $x = 2r$, in which case it will leave the cell altogether, or it will be withdrawn to a point lying between $x = r$ and $x = 2r$, in which case we have to consider the remainder of the cycle of rotation and the cycle of rotation which follows. For the remainder of the rotation the particle will be drawn inwards because

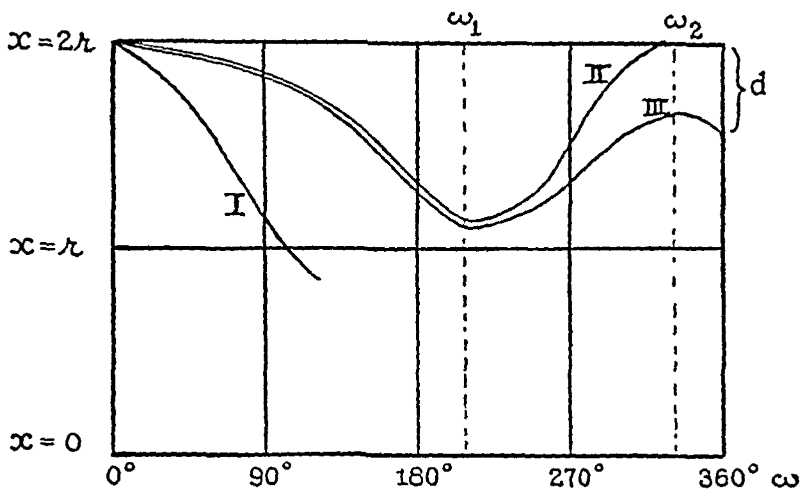

FIG. 3. Graph showing events in the cycle of rotation from $\omega = 0°$ to $\omega = 360°$. In curve I the particle passes to the position $x = r$ in the first two quadrants. In curve II it leaves the cell surface between ω_1 and ω_2, and in curve III it passes into the cell for the small distance d in the complete cycle.

the force F_1 exceeds the force F_λ, and at the end of the cycle the particle will find itself at the junction of quadrants II and I, where it is acted upon by the force F_1 only, as at the beginning. The whole cycle may now occur over again.

The problem will become more clear if the events during the cycle are represented graphically, as in Fig. 3. The ordinates represent values of x, two in particular being indicated, $x = r$, and $x = 2r$. The four phases of the cycle are shown by vertical lines corresponding to angles of rotation ω, the rotation being estimated from $\omega = 0°$ at

the junction of quadrants II and I. The two critical values ω_1 and ω_2 are also indicated in quadrants III and II respectively, and values of t are inserted to show the time relation of the rotation, it being assumed for convenience that a complete rotation occurs in time $2t$. Curves indicate three essential types of happening, dependent on the value of the constants involved in the process.

Curve I.—Here the particle starts at $x = 2r$ at $t = 0$ and $\omega = 0°$, and is drawn inwards according to expression (20). The maximum velocity is reached at $t = 0.5$ or $\omega = 90°$. Before the point $\omega = 180°$ the particle reaches the position $x = r$, and therefore must be completely ingested in the course of time irrespective of the rotation of the cell, for there are no forces to pull it out.

Curve II.—The particle starts at $\omega = 0°$ at $x = 2r$ and is drawn into the cell until $\omega = 180°$. For the short period between $\omega = 180°$ and $\omega = \omega_1$ it is still drawn in, but with a decreasing velocity since the force is decreasing, and at the point $\omega = \omega_1$ it begins to be withdrawn. It is withdrawn more rapidly than it passes in, and before the point $\omega = \omega_2$ it reaches the position $x = 2r$ and leaves the cell surface altogether.

Curve III.—Again the particle starts at $\omega = 0°$ at $x = 2r$, and passes into the cell until $\omega = \omega_1$. Between the points ω_1, ω_2 it is withdrawn, but does not reach the position $x = 2r$ by the time the rotation has proceeded to $\omega = \omega_2$. Under these circumstances it finishes the cycle a little further into the cell than it was at the commencement of the cycle, and on the cycle being repeated sufficiently often it will ultimately be completely ingested.

To put the matter in as simple a form as possible, the ultimate fate of the particle is very largely dependent on whether or not it reaches the point $x = r$ before the half rotation is completed in time t. If it does, it will be permanently ingested; if it does not, it may not be ingested at all. Now, if the velocity of motion of the cell, the surface tension conditions, and other subsidiary factors which enter into the equations of motion are fixed, whether this passage takes place in time t will depend on the cytoplasmic viscosity, the lower this is the greater the chance that the conditions for ultimate ingestion will be established. If, on the other hand, the cytoplasmic viscosity is exceedingly high, the particle will find itself very nearly at $x = 2r$

when $\omega = \omega_1$; during the period from $\omega = \omega_1$ to $\omega = \omega_2$ it will be exposed to the forces which withdraw it, and will amost certainly pass away from the cell surface altogether. Against this may be objected that the resistance to outward motion depends on the vicosity of the cytoplasm also, but it is to be remembered that k_4 is certainly greater than k_3.

To put the different types of result illustrated by these three curves into a general form which will tell us what will happen to a particle under any circumstances is almost impossible, especially when it is remembered that the particle may make contact with the cell surface at any point, and only very improbably at the point $\omega = 0°$. The general conclusions are, however, perfectly clear, and may be set down.

(*a*) The greater the cytoplasmic viscosity, the less likely is it that particles will be ultimately ingested. This is, of course, a well recognised fact, but the reasons for it are much more complex than is generally believed. The result depends principally on the fact that a high cytoplasmic viscosity leaves the particle more liable to removal by forces generated by the movements of the surrounding fluid.

(*b*) The more rapid the rotation of the cell, *i.e.*, the more irregular the motion of the fluid surrounding it (for when there is irregular motion the direction of the movement of the surrounding fluid is constantly changing) the less probable will it be that the particle will be ingested. In the same way, ingestion is less likely to occur in a fluid in which the cells are moving with great velocities relative to their surroundings, as in a violently agitated tube. In this statement it is of course to be remembered that the likelihood of ingestion occurring is to be considered as less only after due allowance has been made for any greater number of collisions which may occur between cell and particle when the movement of the fluid is rapid or irregular.

In conclusion it must be admitted that the results of this investigation are singularly unsatisfactory, for they are only of the nature of approximations, they are incapable of experimental verification except in a very general way, and their importance is quite out of proportion to the difficulty of obtaining them. The justification for calling attention to them lies merely in the fact that they, or results

like them, must ultimately find a place in the complete physical theory of phagocytosis which will some day be developed, together with the fact that they serve to throw some light on the nature of the process of ingestion as it must occur in experiment.

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HEMOLYSIS BY SAPONIN AND SODIUM TAUROCHOLATE, WITH SPECIAL REFERENCE TO THE SERIES OF RYVOSH.

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This paper is concerned with the investigation of the occurrence of the resistance series known as the series of Ryvosh, when (*a*) saponin and (*b*) sodium taurocholate are employed as hemolytic agents.

The essential literature on the subject is readily summarised. Ryvosh (1907), using non-quantitative methods, has investigated the hemolysis of the cells of certain animals by saponin and by hypotonic saline solution. The series of Ryvosh consists of these animals arranged in the order of the resistance of their cells to saponin hemolysis, which order is the reverse of that obtained when the same animals are arranged according to the resistance of their cells to hemolysis by hypotonic saline. This series has been reinvestigated and confirmed by Ponder (1927) who has employed a strictly quantitative technique. Kofler and Lázár (1927), making use of different and less exact methods, have been able to confirm the series of Ryvosh for hemolysis by saponin. Using other glucosides they have failed to obtain the series. Finally, Ponder and McLachlan (1927) have obtained values of the resistance constant, *R*, for several of the animals included in the series, using hemolytic agents of bacterial origin. They have concluded that the values so obtained are not sufficiently different to justify the arrangement of the various animals in a series. The question arises as to whether the series of Ryvosh is of general applicability or whether saponin may be considered as a special hemolytic agent in this respect. It is of special importance to extend the investigation of the series of Ryvosh to other hemolytic agents, as yet not utilised in this respect, and with this in mind the following experiments with saponin and sodium taurocholate were carried out.

I. The Series of Ryvosh and Saponin Hemolysis.

Method.

The method used in the experiments of both parts of this paper and in the analysis of all the curves is essentially that developed and fully described by Ponder (2–6). These papers contain a complete explanation of the procedures, which will be briefly described here.

The dilutions of lysin are prepared in such a way that when the lysin is added to the systems the final dilution of the hemolytic agent is that desired. With the exceptions of the sheep and the ox, in which cases the dilutions used were one-tenth as great, the final dilutions used in the experiments with saponin were 1 in 10,000 to, usually, 1 in 60,000, *i.e.*, 1 part of saponin to 10,000 parts of 0.85 per cent NaCl. The test-tubes used were small white glass tubes, 4 by 0.5 inches. These were thoroughly cleaned, finally steamed with live steam and then dried in an oven. To secure a single point on the experimental curve, 0.8 cc. of the lysin dilution is placed in a test-tube and to this is added 0.8 cc. of 0.85 per cent NaCl. The tube is then placed in a glass-sided water bath having a white illuminated background and kept at a constant temperature of 25°C. In the water bath is also kept the blood suspension and the pipette used to transfer the suspension to the test-tube. Time being allowed for these to all acquire the temperature of the bath, the hemolytic system is completed by the addition of 0.4 cc. of the blood suspension, the blood corpuscles of which are kept in suspension by frequent shakings. The blood suspension used is the "standard" suspension of Ponder, consisting of the corpuscles from 1 cc. of oxalated blood, twice washed and suspended in 20 cc. of 0.85 per cent NaCl. With a stop-watch the time is taken for complete hemolysis to occur. This time plotted against the corresponding dilution gives one point on the experimental curve.

In each experiment two such time-dilution curves are secured, one for man, taken as a standard, and one for the animal whose relative resistance to the lysin is being investigated. The times of complete hemolysis corresponding to a dilution of, say, 1 in 10,000 are taken simultaneously for the cells of man and of the animal. These two times are then plotted against the corresponding dilution and the first point on each of the two experimental curves thus obtained. The procedure is then repeated in order to secure the times corresponding to the next higher dilution of the lysin, *i.e.*, 1 in 20,000, etc.

To illustrate the method used in the analysis, the curves for rabbit and man may be taken as an example. The two curves are plotted together upon the same piece of graph paper. A single point on each curve corresponding to a certain time is noted. Twice the reciprocal of the dilution thus secured in the case of the human cells is taken as equal to c_1 and twice the reciprocal of the dilution for the rabbit cells taken as c_2. In the same way the values of c_1 and c_2 are obtained for several other pairs of points on the two curves. When the c_1 values are plotted against the c_2 values an approximately straight line should be secured. Ponder (4) has discussed this c_2/c_1 ratio and has pointed out that the curves ob-

tained by plotting c_1 against c_2 in this type of experiment is not a straight line but that for all practical purposes it may be taken as such. Having thus determined the straight line, the best value of R, the resistance constant, is obtained from the ratio c_2/c_1 and R is taken to be constant for all corresponding points on

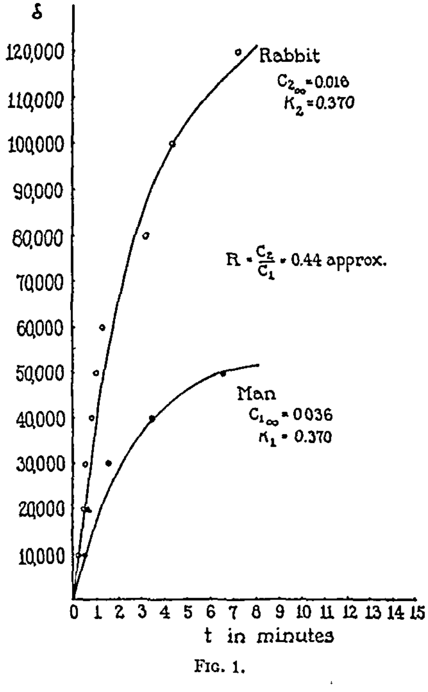

FIG. 1.

the two curves. The theoretical curves are then fitted to the experimental points.

The equation of the time-dilution curves is the well known first order expression

$$t = \frac{1}{\kappa} \log \frac{c}{c - x},$$

where c is the initial concentration of the lysin, x is the amount of lysin used up by the system at any time, t, and κ is a composite constant. The value of c for any dilution, δ, is obtained by taking twice the reciprocal of the dilution and is in milligrams. When $t = \infty, x = c_\infty$, *i.e.*, the total amount of lysin that the system uses up. In practice the value of x is determined by doubling the reciprocal of the dilution corresponding to the asymptote of the curve. The value of log $c/(c - x)$ is then calculated for each observed time and the value of κ derived from that theoretical curve which best fits the experimental points. Table I gives the values of c, the number of milligrams of lysin in the system, the observed values of t and the calculated values of t for the rabbit and for man. R for this pair of curves is approximately equal to 0.44.

TABLE I.

c	Man		Rabbit	
	t obtained	t calculated	t obtained	t calculated
	min.	*min.*	*min.*	*min.*
0.2	0.5	0.54	0.3	0.23
0.1	0.6	1.20	0.5	0.47
0.06·	1.5	2.13	0.5	0.75
0.05	3.5	3.44	0.8	1.04
0.04	6.5	6.2	1.0	1.38
0.03·			1.3	1.76
0.028				
0.025			3.2	2.76
0.022				
0.020			4.3	4.35
0.018				
0.017			7.2	7.65

In Fig. 1 the theoretical curves are represented by lines and the experimentally determined points as small circles (rabbit) and large dots (man). The second point in the curve of man, represented by a triangle, obviously results from an error. In both curves the experimental points corresponding to the higher concentrations fall inside the calculated curves. That this is a general occurrence has been pointed out by Ponder (4). With the exception of the two points mentioned, both experimental curves are found to fit the calculated ones sufficiently satisfactorily for the purposes of this paper.

The saponin curves have been analysed and the resistance constant, R, determined for each animal of the series. These values have been correlated with the values of R obtained by Ponder (5) according to the expression

$$r = \frac{S\ (xy)}{\sqrt{S}\ (x^2) \cdot \sqrt{S}\ (y^2)},$$

where r is the coefficient of correlation, x and y deviations from the mean of the values of R in the two series and $S(x^2)$ and $S(y^2)$ the sums of the squares of the deviations.

Results.

The results of these experiments may be briefly expressed as in Table II, which shows the values of R given by Ponder (5) and the values of R derived from the present experiments.

The coefficient of correlation between these two sets of values has been found to be 0.89. Considering that the animal described as "goat" was *Capra hircus* in Ponder's series and *Hemitragus jemlaicus* in this series, the value of r is a very good one. These two series

TABLE II.

Animal	Ponder	Author
Rabbit	0.4	0.44
Rat	0.7	0.7
Pig	1.3	1.0
Man	1.0	1.0
Guinea pig	1 1	1.1
Dog	1.2	1.65
Cat	2.1	4.2
"Goat"	2.5	5.0
Sheep	7.0	6.0
Ox	6.6	7.3

were obtained, one by Ponder in Edinburgh and the other by the author in New York. It is of interest that similar results have been given by two so widely separated groups of animals.

It may be concluded that the series of Ryvosh is obtained when saponin is used as the hemolysing agent.

II. The Series of Ryvosh and Sodium Taurocholate Hemolysis.

Method.

The method used in the experiments with sodium taurocholate is essentially that described in the first part of this paper, such modifications as occur being necessary because of the fact that sodium taurocholate very rapidly changes its hemolytic properties in solution. These modifications are as follows: (1) while dilutions of saponin will keep on ice for several days, a stock dilution of sodium taurocholate must be prepared at the latest possible moment before use. The

stock solution is such that, if itself used as the hemolytic agent, would give a final dilution of 1 in 100. (2) Unlike the saponin system, the sodium taurocholate system necessitates rapid working; for each pair of points corresponding to the curves of man and of the animal the dilution must be made individually from the stock solution and the two points determined simultaneously. With saponin, however, the time interval between the preparation and the use of the dilutions need not be at a minimum and so a number of tubes may be allowed to hemolyse together. (3) It is even more important with sodium taurocholate than with saponin that the glassware used be thoroughly cleaned and dried, especially the test-tubes which are to contain the hemolytic systems.

The analysis of the curves is carried out in the same manner as in the case of the saponin curves, with the following exceptions. Plotting c_1 against c_2 does not result in a straight line, since the two curves compared are found to approach their respective asymptotes with different values of κ. The resistance constant, R, has therefore been determined from the ratio

$$\frac{c_{2\infty}}{c_{1\infty}} = R_\infty.$$

R_∞ furnishes no information concerning the velocity constants, κ_2 and κ_1, of the curves of the animal and man respectively. Unlike saponin hemolysis, sodium taurocholate hemolysis yields curves having different values of the velocity constant. The values of κ may be quite different for two comparable curves. Further, κ_1 is found to have different values in different experiments. There are several reasons for attributing the latter variation to changes in the hemolytic properties of the sodium taurocholate while in solution. In the first place, it is well known that the bile salts are very unstable in solution, undergoing changes which affect their ability to hemolyse red cells. Secondly, it has been found difficult to make the time interval between the preparation of the stock solution of taurocholate and its use the same for each of the experiments. Thirdly, the blood of man used was always taken in the same manner from the same individual and the standard suspension was made up in the same way in each experiment, thus greatly reducing the probability that changes may have occurred in the cell component of the system. It is therefore desirable to have an expression of the velocity constant of the curves of the series which will enable the values of κ_2 to be more readily compared. This may be done by taking the ratio

$$\frac{\kappa_2}{\kappa_1} = A.$$

Better relative values of κ_2 are offered by A than by κ_2 itself.

A typical example of the time-dilution curves obtained from the sodium taurocholate experiments is that seen in Fig. 2 for the cells

of the rabbit and of man. In Table III are shown the concentrations of the lysin and the corresponding observed and calculated times for each of the curves. Fig. 2 shows the experimental and calculated curves. It is to be noted that the two curves cross as they approach

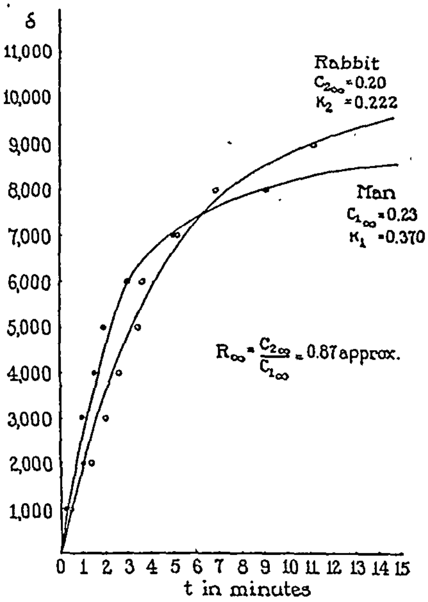

FIG. 2.

their respective asymptotes; crossing has not been found to occur in every case but the curves compared always show different values of κ.

Results.

The results of the experiments with sodium taurocholate may be briefly expressed. Table IV gives the values of R derived from the

saponin experiments, the values of R_∞ obtained from the taurocholate experiments and the values of A. It should be remarked that the values of R_∞ do not vary greatly from unity. In determining A, κ_1 is taken equal to 1, but the experimental values of κ_1 have been found to vary from 0.37 to 0.43 with a mean of 0.40 ± 0.32.

TABLE III.

c	Man		Rabbit	
	t obtained	t calculated	t obtained	t calculated
	min.	*min.*	*min.*	*min.*
2.0	0.2	0.33	0.4	0.47
1.0	1.0	0.7	1.4	1.0
0.6	1.0	1.13	2.0	1.58
0.5	1.5	1.7	2.6	2.3
0.4	1.9	2.3	3.4	3.12
0.3	2.9	2.9	3.6	4.1
0.28	4.9	4.7	5.0	5.6
0.25	9.0	8.7	6.8	7.2
0.22			10.5	10.8

TABLE IV.

Animal	$R = \frac{c_2}{c_1}$ (Saponin)	$R_\infty = \frac{c_{2\infty}}{c_{1\infty}}$ (Na taurocholate)	$A = \frac{\kappa_2}{\kappa_1}$.
Rabbit	0.44	0.87	0.60
Rat	0.7	1.00	0.62
Pig	1.0	1.09	0.36
Man	1.0	1.00	1.00
Guinea pig	1.1	0.64	0.28
Dog	1.65	0.85	0.36
Cat	4.2	1.05	1.52
"Goat"	5.0	0.78	0.34
Sheep	6.0	0.76	0.25
Ox	7.3	1.00	0.24

The coefficient of correlation for the values of R and R_∞ has been calculated as before and r found to be approximately −0.21, a correlation value of no significance in this connection.

It must be concluded that the series of Ryvosh is not obtained when

sodium taurocholate is used as the hemolytic agent and when R_∞ is taken to represent the resistance constant, R. This increases the probability that saponin may be a special hemolytic agent in so far as it can produce the series of Ryvosh and adds sodium taurocholate to the group of lysins, exemplified by bacterial lysins and a number of glucosides other than saponin, which give different resistance series (7, 9).

SUMMARY.

1. The series of Ryvosh is obtained when hemolysis of the red cells of the animals concerned occurs with saponin as the lytic agent.

2. The series of Ryvosh is not obtained when R_∞ is taken as the resistance constant and sodium taurocholate is used to hemolyse the cells of the same animals.

3. The hemolysin sodium taurocholate has been found to differ from saponin in that the time-dilution curves are found to approach their respective asymptotes with different values of κ.

The author wishes to acknowledge indebtedness to the New York Zoological Society, and especially to Dr. Charles Noback of the New York Zoological Gardens, for the blood of *Hemitragus jemlaicus*.

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ON THE GEOTROPIC ORIENTATION OF YOUNG MAMMALS.

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I.

An account of geotropic orientation in young rats and mice has been given in several preceding papers.[1] The points made were essentially these: upon a plane inclined at angle α with the horizontal the path of steady progression is at a mean angle θ to the intersection of the plane with the horizontal; the magnitude of θ is roughly proportional to $\log \sin \alpha$; more precisely, $\cos \theta$ is a linear function of $\sin \alpha$; the speed of progression obeys similar rules. These relationships were tested by a large number of observations, and specifically by the composition of fields in which the geotropic excitation was exactly counter-balanced by phototropic stimulation,[2] and by means of the effect of masses attached to the base of the tail. The precision with which such data may be obtained, under good conditions of temperature, age of animals, absence of photic stimulation, and selection of vigorous litters from among genetically uniform families, encouraged the attempt to investigate by their aid the possible relations in heredity of quantitative aspects of conduct. It was found that representatives of inbred lines belonging to different species of rats gave results agreeing with the equations already stated, but with various values of the respective constants.

In the papers previously published[1] we have relied upon measurements made with homogeneous groups of small numbers of individuals, although stating that these were given as illustrative. For the proposed investigation, however, it was first necessary to discover if with animals of a given strain, initially well inbred, the same values

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[1] Crozier and Pincus (1926; 1926–27, *a*, *b*, *c*); Pincus (1926–27); Crozier and Oxnard (1927–28); Keeler (1927–28); Crozier (1928).

[2] Crozier and Pincus (1926–27, *c*).

of the descriptive constants could be recovered with successive generations. We then sought other strains in which either the slope of the relation between θ and α might be different, or the magnitudes of the intercepts on the axes of the *cos* θ *vs. sin* α plot, or both. It was found that the values of the constants in the strain initially used are indeed recoverable at intervals over 15 months; and that strains differing in the desired ways are in fact procurable.

II.

The families of *R. norvegicus* first used[1] were of a backcross stock between King inbred albinos and a dark-eyed (hooded) stock, labelled line *K*. This line was continued, and in two groups of experiments series of tests were again made, at different periods and with slight differences of technic, to determine the relation of the amount of orientation to the inclination of the creeping surface. The first extensive repetition of the original tests was made by a method slightly different from the one previously followed. At the beginning of each run a rat, 13 days after birth and with eyelids still unopened, was placed in a dark room at 19°–21°C. upon a large horizontal plane covered with tightly stretched wire screen of small mesh. The plane was hinged at one margin to a rigid support. When the effect of handling had more or less worn off, and the animal had begun to creep, the plane was slowly brought to a predetermined inclination to the horizontal. At the desired angle the lateral margin of the plane was held by a notched tongue of wood. In slipping into a notch a slight jarring of the plane was unavoidable, and the creeping of the rats seemed in consequence unusually variable, by comparison with series employing the older technic. The average values of θ agreed quite well with those earlier obtained, though the variability was somewhat greater, proportionately; the slope of the best line connecting θ and log *sin* α was practically identical with that in previous tests, but the values of θ were consistently a little lower. This was probably due in part to the lower temperature (20° ± 1.0) prevailing in the later tests (*cf.* 23°–25° in Series I, III, etc.).

The newer technic was improved by eliminating the "catch" mechanism and substituting for it a stout cord working over a smooth pulley. The cord could be held fast at definite points corresponding to known elevations of the plane.

A series of tests with one litter of four individuals is summarized in Table I. The averages are here based upon 40 records of orientation at each value of α, as in the earlier published series of measurements with 2 individuals. These conditions were chosen for the sake of a test shortly to be applied to the measurements of variability of θ.

The manner in which the averages in Table I agree with the results of typical earlier tests is evident in Fig. 1. Here the series with line *K* already published (Crozier and Pincus, 1926–27, *a;* a confirmatory series is given in Pincus, 1926–27) is labelled "Series I," the present test (Table I) is "Series III." (Series II, previously referred to,

TABLE I.

Mean angles θ of paths of negatively geotropic orientation of 4 individuals (litter mates) of *Rattus norvegicus*, line *K*. The inclinations of the creeping stage are given in the first column.

α	θ
20°	43.70° ± 2.05
25°	53.73° ± 2.24
30°	55.98° ± 1.72
35°	62.50° ± 1.21
45°	69.89° ± 1.18
55°	81.80° ± 0.81
70°	86.76° ± 0.43

agrees well with these but the P.E. are too large, owing to the low temperature and to the mechanical disturbances there connected with elevating one edge of the creeping platform.) In Fig. 1 it is apparent that to a reasonable approximation $\Delta\,\theta/\Delta\, \mathit{log\ sin}\ \alpha$ is constant over the whole working range. But it is equally clear that, with these coordinates, the curve is in reality a long-drawn *S*. Every series of tests has shown this to be true. The meaning of this relationship will be discussed in a different connection.

As demonstrated previously,[1] the function involving θ and α which gives a rectilinear plot is $\Delta \cos \theta/\Delta \sin \alpha = \mathit{constant}$. Measurements of Series I and III are given in this form in Fig. 2.

III.

It has been objected (Hunter, 1927) that successive readings of θ in such experiments may give bimodal frequency distributions, or

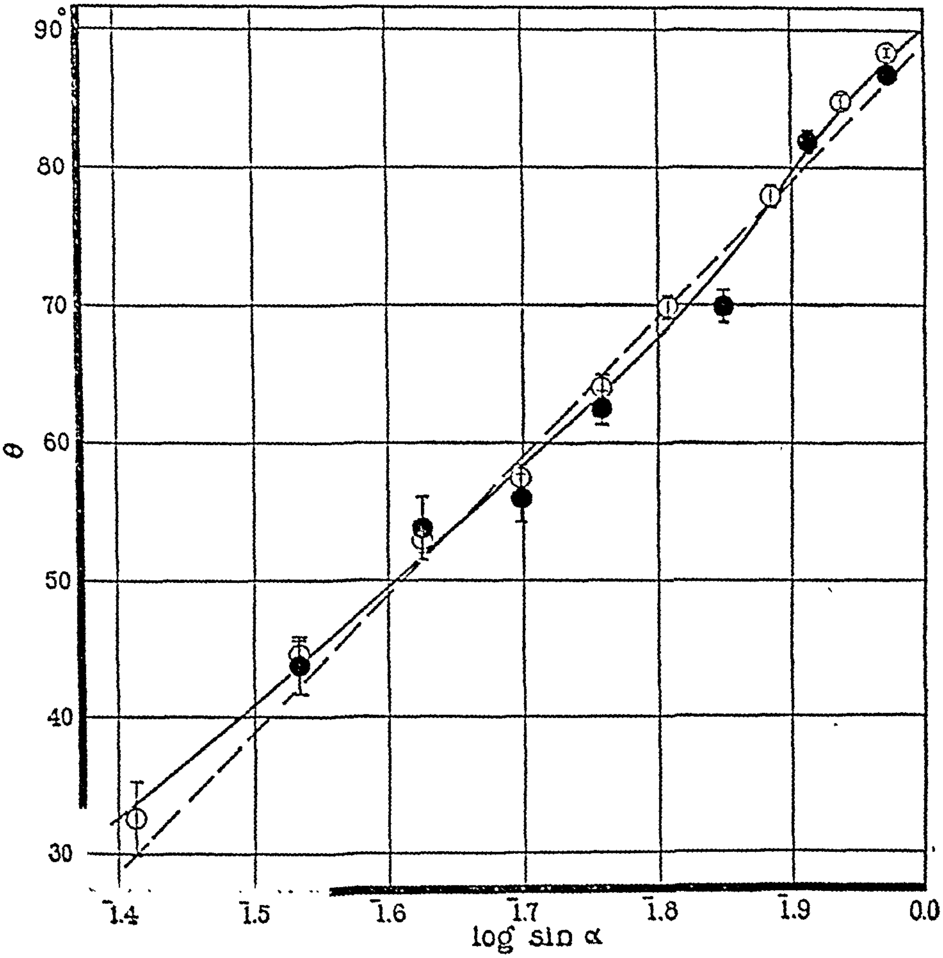

Fig. 1. Showing the agreement in the relation between amplitude of negatively geotropic orientation θ and *log sin* α, where α is the angle between plane of creeping and the horizontal, in two series of tests with young rats (*R. norvegicus*, line *K*; see text) made 15 months apart. The height of the vertical bars = 2 P.E. For certain purposes the relation may be regarded as effectively rectilinear, though in reality sigmoid.

distributions quite asymmetrical. If proper and reasonable precautions are taken, and attention paid to the geometry of + and − θ,

we have found this not to be true. Certain effects which may produce such results have already been commented upon (Crozier, 1928; Crozier and Stier, 1927–28). For the present data, Series I and III, we may as a direct test consider certain properties of the measured

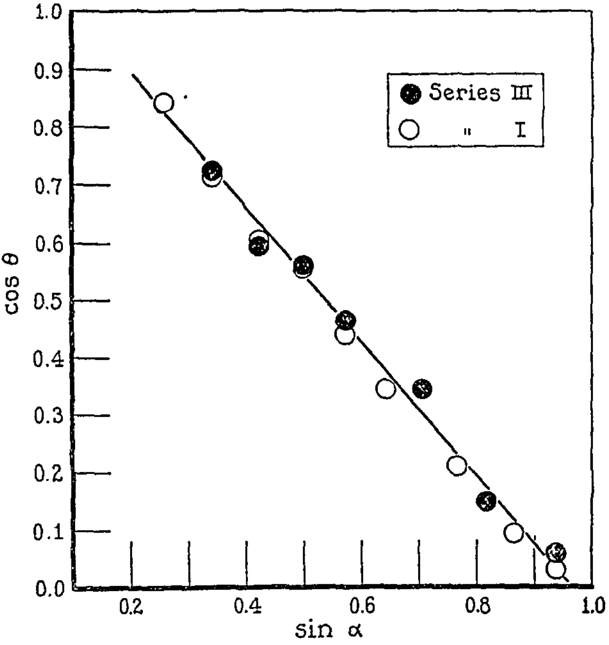

Fig. 2. The agreement of two independent series, I and III, in terms of a linear connection between *cos θ* and *sin α*. See text.

variability of θ in the two series, and also the relation between *median* magnitudes of θ from combined frequency distributions of Series I and III. Figs 3 and 4 show that the median values of θ are related to

log sin α, and of cos θ *med.* to *sin* α, exactly as are the means (Figs. 1 and 2). The evidence from the P.E. of θ is perhaps not so obvious, but is even more powerful. We already know that *C.V.* θ (or, n being constant, P.E. $\times$ 100) decreases rectilinearly with *log sin* α.

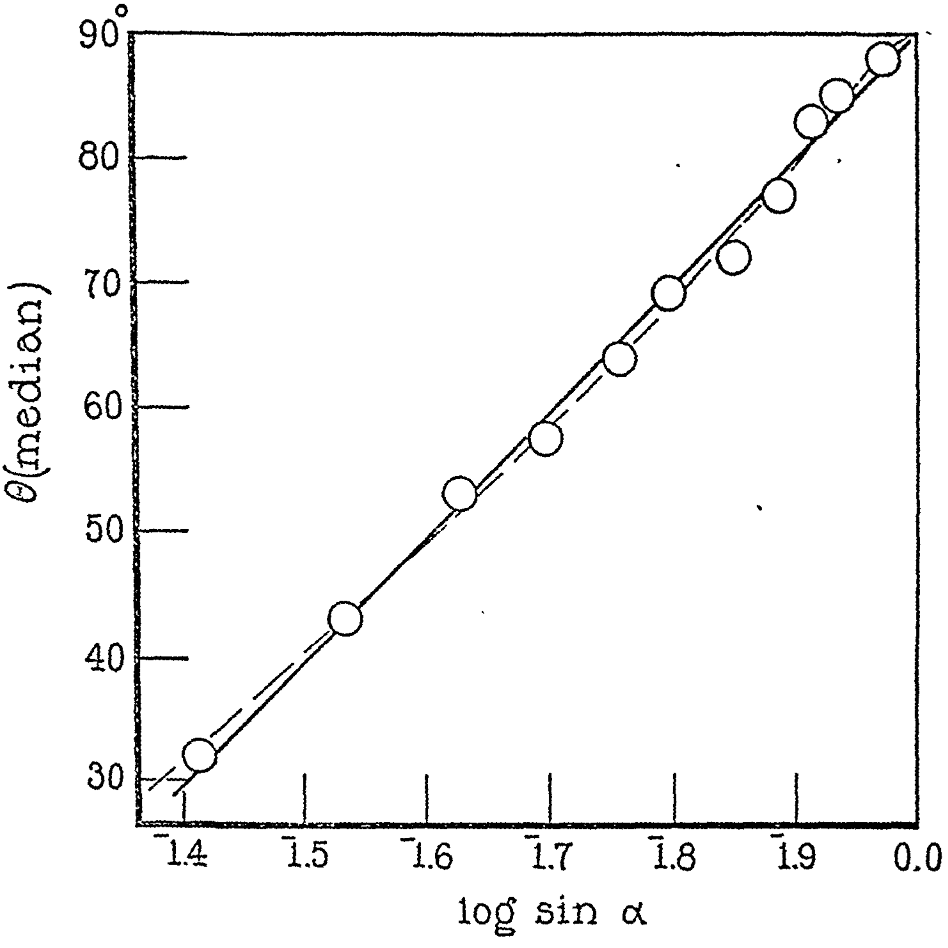

FIG. 3. θ *median* from combined distributions of Series I and III bears the same relation to *log sin* α as does mean θ.

If in the course of the experiments two litters of like individuals are compared, the same number of observations (n) being taken in each set at each value of θ, but with more individuals in one set than in the other, then for the set with fewer individuals the relative variability should be found lower at a given value of α, but should decrease *less*

rapidly as α increases. The point is that if the experiments are properly conducted the method of averaging should eliminate all but "chance" values of θ due to momentary individual causes; hence, since $\Delta\ C.V./\Delta\ log\ sin\ \alpha$ is constant, and the gravitational vector must

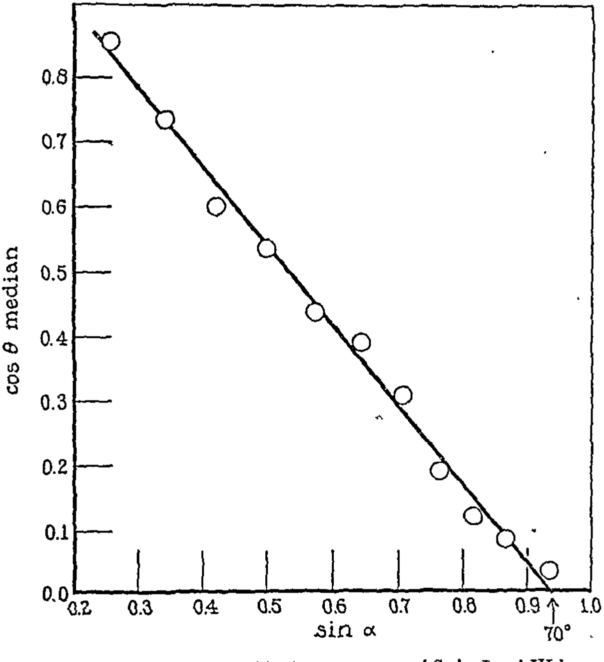

FIG. 4. *Cos θ median* from combined measurements of Series I and III bears the same relation to *sin α* as does *cos θ mean*.

therefore be regarded as eliminating change influences of external forces capable of affecting the orientation, and according to exactly the same law as that by which θ itself is controlled, the variability

of internal origin bulks relatively twice as large in a series with twice the number of individuals. Fig. 5 shows that in Series III, with 4 individuals, the decline of $\frac{100 \times \text{P.E.}}{\theta}$ with increasing *log sin* α is almost exactly twice as rapid as with Series I (2 individuals).

IV.

Of great importance for the objective of these experiments is the finding of races of rats or mice in which the constants in the equations

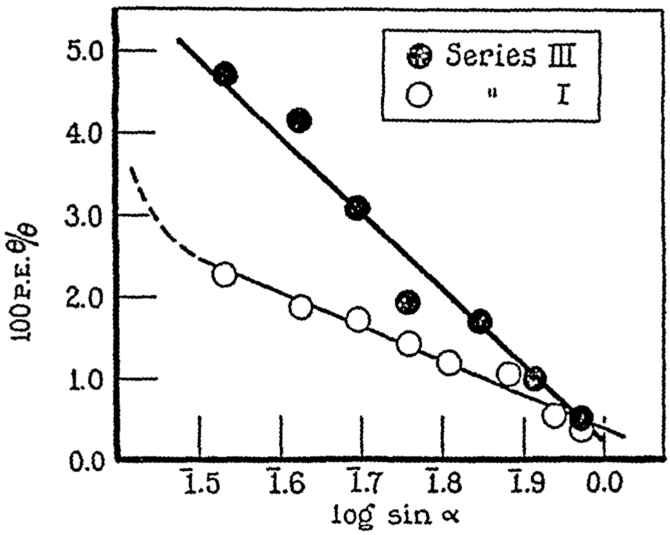

FIG. 5. The variability of θ, expressed by P.E. θ as a percentage of the mean (the number of variates being 40 in each case) declines directly as *log sin* α increases. The rate of the decline is directly proportional to the number of individuals concerned: in Series I, 2 individuals, in Series III, 4, the slopes of the lines being in the ratio 1:2.1.

describing geotropic orientation are different from those found in the race thus far used. The genetic utilization of such differences, supposing them to be found, can provide upon the one hand a biological test of the reality of the behavior-constants, and upon the other a means for the consideration of certain concrete aspects of behavior in relation to inheritance. Two kinds of difference might be expected among races for which the general formulation already used is applicable. The slope of the rectilinear relationship between *cos* θ and *sin* α

might be different; or, with equivalence of slope, the intercepts might not be the same. With *R. rattus* it was in fact found (Crozier and Pincus, 1927–28, *d*) that the constants in the equations differed from those gotten with *R. norvegicus*. Experiments with several strains

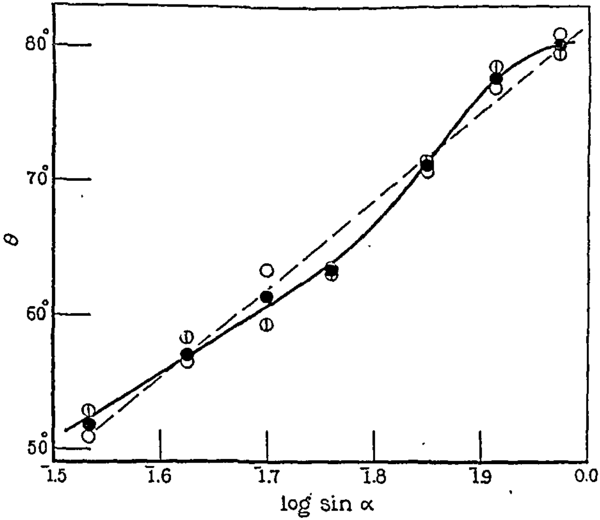

FIG. 6. Upward geotropic orientation of two series of rats of strain *A* (see text); Nos. 1–6, open circlets; Nos. 7–12, circlets with bar; means, 1–12, solid circlets. The ordinate scale is relatively larger than in Fig. 1, and the *S*-form of the relation between θ and log sin α is therefore more pronounced; the linear proportionality between θ and log sin α is still sufficiently close for some purposes.

have since shown that, independently of possible connections with body weight or slight differences of age, it is possible to obtain strains of rats and of mice in which both kinds of difference from *R. norvegicus*, strain *K* (Crozier and Pincus, 1926–27 *a;* and Pincus, 1926–27), are definite and specific. The strain most extensively used for compari-

son, labelled *A*, is characterized here in illustration of this point. This particular strain has been used with strain *K* in genetic experiments of the sort earlier forecast (Crozier and Pincus, 1926–27, *d*); the results will be considered in a subsequent paper. Strain *A* is a red-eyed yellow hooded race which had been bred brother to sister for ten generations after previous loose inbreeding.

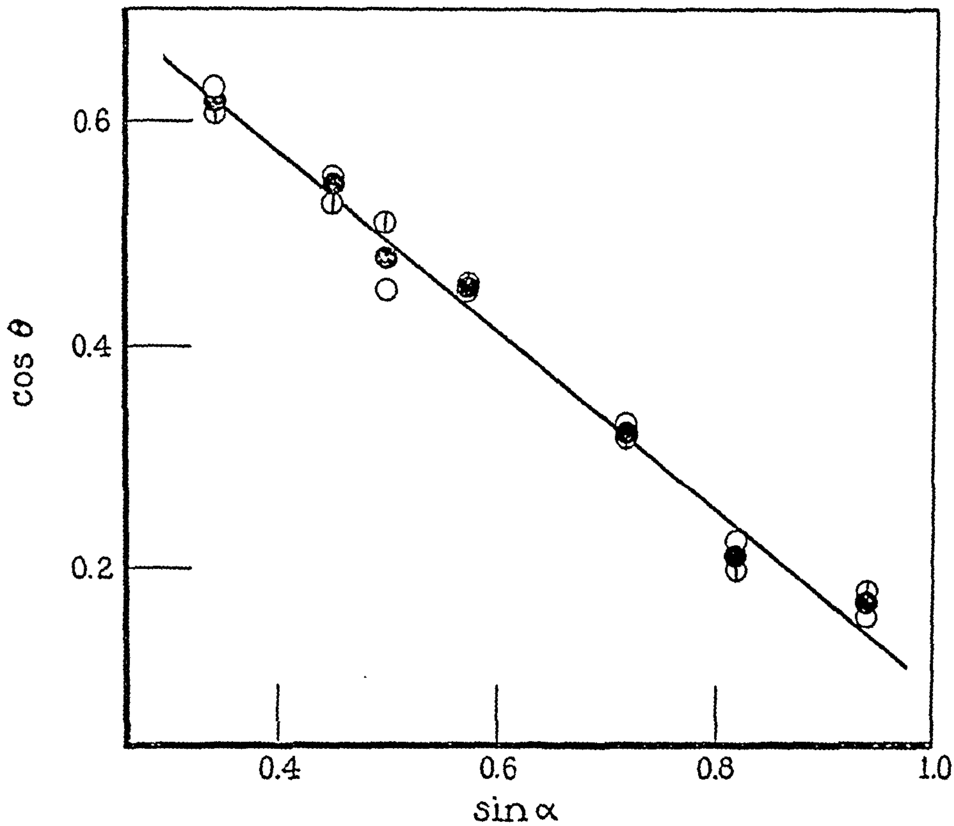

FIG. 7. The relationship between cos θ and sin α for strain *A*; individuals 1–6, open circlets; 7–12, circlets with bar; general means, solid circlets.

Occasional temporary geopositive orientation is observed in both the strains here considered. Such geopositive orientation is also amenable to quantitative treatment, but we do not attempt this at present. The initiation of a geopositive phase is marked by peculiar sidewise creeping during which the animal moves upward at a steadily decreasing angle, so that its path forms a curve. It may move hori-

zontally for a while, but, in any such case, it eventually turns downward and moves at an angle which is about equal to the angle (θ) of geonegative orientation characteristic of the angle of inclination employed. The change to a geopositive phase is almost always heralded by the peculiar sidewise movement described, during which the animal seems to be struggling between a tendency to move upward

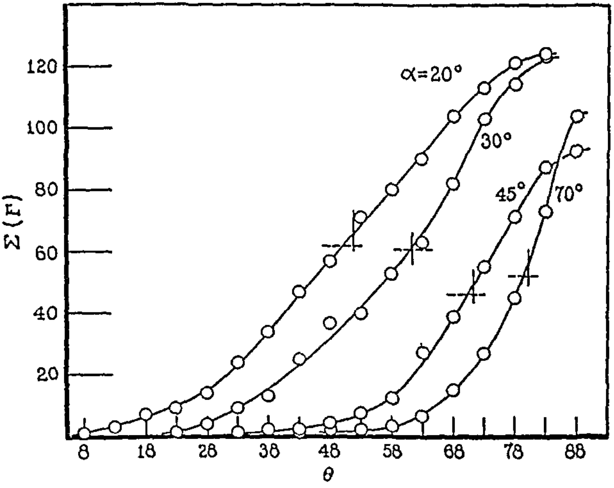

FIG. 8. Frequency distributions (line A, 12 individuals) of θ at several values of α. See text.

and an almost equal tendency to move downward. (Geopositive orientation seems to occur more frequently at lower angles of inclination.) Such orientation was found to occur much more frequently in line A than in line K. It is noteworthy in this connection that for line K measurements of θ can be taken with some reliability at $\alpha = 15°$, but that this is impossible with A. Thus there may be some connection between this greater sensitivity of line K to geotropic stimulation

(note also the steepness of the slope of the curve (Fig. 9)), and its infrequent geopositive orientation.

Two comparable litters of the tenth inbred generation of *A*, containing 6 individuals each, were tested independently. The weights, on the 12th day for Nos. 1–6, 13th day for Nos. 7–12, were respectively 13.0 to 15.5 gm. and 15.5 to 18.0 gm. As in earlier tests, there is no apparent correlation of geotropic performance with body weight. Differences of 2 days in age are likewise of no significance, as other experiments with individuals on successive days amply prove. Average

TABLE II.

Geotropic orientation of young rats of race *A*, 10th inbred generation (see text); 12 individuals, in 2 series of 6 each (the 2 series given separately in Fig. 6). The means from the single individuals being comparable, all are averaged together. The nature of the frequency distributions is indicated in Fig. 8. α = inclination of the surface, θ = angle of upward orientation, n = number of observations.

α	n	θ
20°	125	51.88° ± 0.23
25°	118	56.97° ± 0.21
30°	123	61.39° ± 0.18
35°	112	63.19° ± 0.16
45°	92	71.24° ± 0.14
55°	113	77.69° ± 0.16
70°	109	80.17° ± 0.10

values of θ derived from trials with rats one to six and seven to twelve are plotted in Figs. 6, 7. The two series again demonstrate the kind of concordance obtainable in these experiments. The average magnitudes of θ from all observations are summarized in Table II. With so many individuals in one series, it is worth while to refer to the frequency distributions of θ at constant values of α. The distributions (as in Fig. 8) are slightly skewed, with a "tail" of low values of θ. Modes and medians are close together. As with other series of measurements, we can expect that the observed frequencies of negative departures from the mode should descrease almost logarithmically with the extent of the departure. This should appear most clearly

at intermediate values of α, since at low values of α the "normal" spread of θ is wide.[3]

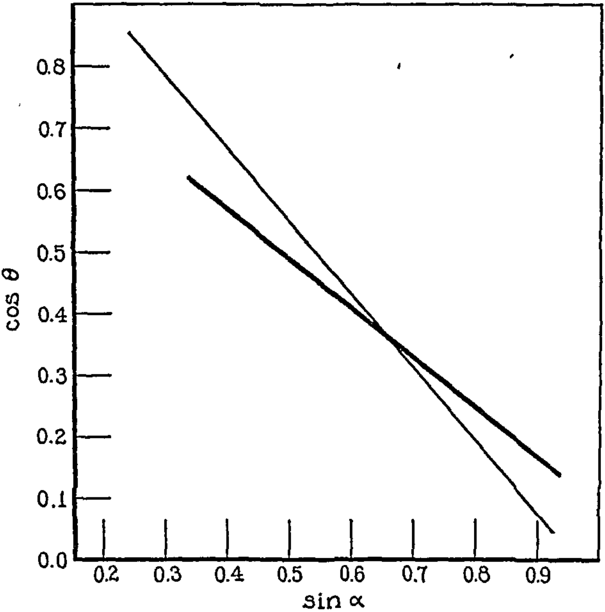

FIG. 9. Comparison of the lines connecting cos θ and sin α, for *R. norvegicus* line K and line A. Data from Figs. 2 and 7. See text.

Comparison of lines K and A is most easily made through Figs. 2 and 7. In Fig. 9 the lines fitting cos θ vs. sin α have been reproduced on one grid. The slopes are quite different, and the lines moreover

[3] On this basis it is possible to institute tests of series of such measurements, in a way which may be employed to discover the presence of extraneous (non-gravitational) excitation factors.

cross at $\alpha = 35°$. This means that in comparing the geotropic reactivity of the two lines it is absolutely necessary to obtain data over the whole possible working range of α. It is obvious that if the differences between lines K and A correspond to heritable differences, litters obtained by simple systems of crossing between K and A should be expected to exhibit considerable variability in the measurable relations of θ to α. On the other hand, these differences can be made the basis of a genetic analysis.

SUMMARY.

Constants in equations of curves describing the geotropic orientation of young rats are repeatedly obtainable from litters of successive generations of the same inbred strains. Different inbred strains have been obtained for which the respective constants are quite unlike.

Such findings show how necessary it is to employ biologically uniform material in experiments of this kind. And at the same time they are convenient as a starting point for genetic analysis.

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GEOTROPIC ORIENTATION IN ARTHROPODS.

I. Malacosoma Larvæ.

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I.

Quantitative description of geotropic orientation as controlled by the magnitude of the effective component of gravity has been obtained for several animals, including young rats (Crozier and Pincus, 1926–27, *a;* 1926–27, *c;* Pincus, 1926–27), mice (Crozier and Oxnard, 1927–28; Keeler, 1927–28), slugs (Wolf, 1926–27; Wolf and Crozier, 1927–28, *a*), and certain others. The interpretation of these descriptions in terms of the processes of excitation which limit the extent of the orientation must be checked through experiments with other organisms, in which particular features of the orientation effect may be separately studied. This is especially the case when it is desired to utilize the descriptions for the investigation of behavior in compound fields of excitation (Crozier and Pincus, 1926–27, *b;* Wolf and Crozier, 1927–28; Crozier and Stier, 1927–28). Ideally, adequate mathematical description must be based upon understanding of the mechanisms governing the orientation. In the nature of the case, these two aspects of the interpretation cannot be approached in ways entirely independent. But by careful examination of structurally diverse animals, creeping in quite different ways, means may be found for the critical testing of the several orientation formulæ. In this connection the distinction must be kept in mind between formulæ which may be empirically adequate and of a type convenient to handle, and rational equations based upon a theory of the controlling mechanism. Thus a linear relationship has been found, with several forms, between $log\ sin\ \alpha$ and θ, where α = the inclination of the creeping plane above the horizontal and θ = the angle of upward orienta-

tion on the plane (Crozier and Pincus, 1926–27, *a;* 1926–27, *b;* Crozier and Oxnard, 1927–28; Wolf, 1926–27). The fit is very good except at quite low values of α. No one would consider the agreement more than an empirically useful one, and as such it has been employed (Crozier and Pincus, 1926–27, *b*). Yet, in a paper containing a variety of other and more curious misapprehensions, Hunter (1927) states that we have found the relationship between α and θ "to follow Weber's Law," although in the paper referred to (Crozier and Pincus, 1926–27, *a*, p. 264) explicit caution against such an idea was quite definite. While certain formulations, of the logarithmic type for example, must not be taken to have more than a useful share of significance, it is important for the uses to which they may be put to demonstrate that they do not result merely from accidents of averaging data as obtained from a number of tested individuals. For this reason we have in crucial cases depended upon data from tests with a few really comparable individuals, although stating (Crozier and Pincus, 1926–27*a*, *b*) that these were cited as illustrative. But proof is given by the fact that with other organisms, where the mechanics of creeping and the possible manner of the limitation of its direction are obviously different, these logarithmic expressions do not apply.

II.

In the experiments already referred to it was found that on a plane inclined at angle α to the horizontal the upward orientation of the actively creeping geosensitive organism is limited to a zone defined on either side by an average angle θ on the plane (*cf.* Fig. 1). The path describing the angle θ is the limiting position for stable progression. The magnitude of this angle is, for first approximation, linearly related to the logarithm of the active gravitational component on the plane, so that

$$\frac{\Delta\,\theta}{\Delta \log \sin \alpha} = const. \tag{1}$$

The precise form of the relationship, and its meaning, will be examined in another place. It is possible to show, also, that in particular cases the striking of the angle θ is determined by the achievement of a

threshold difference between the downward pull of the body upon the musculature of the two sides. Thus in the slug *Agriolimax* (Wolf, 1926–27) it is sufficient to assume that the downward pull of elements of the body mass upon corresponding longitudinal elements of the parietal musculature at the anterior end of the animal becomes so adjusted, at orientation, that the difference in the tensions produced on the two sides is reduced to a constant value. This requires that the longitudinal elements affected be mutually inclined, and at a constant angle ($2\ h$). The trigonometric expression to which these assumptions lead is adequately verified (Wolf, 1926–27); it states that when the limiting angle (θ) is reached and maintained,

$$(\Delta \sin \alpha)(\Delta \sin \theta) = const., \tag{2}$$

and it assumes that the "head angle," $2\ h$, is constant; this assumption might be tested by experiments with larger slugs (*e.g.*, *Limax*) when forced to carry added loads. Another method of investigation is to employ an animal in which the "head angle" is not structurally determined, but is conditioned by the side to side swinging of the anterior end during progression, and in which the amplitude of such swings may be seen to vary. The tent caterpillar *Malacosoma americana*, and certain other larvæ, provide suitable material. The negative geotropism of such beetles as *Tetraopes tetraopthalmus* affords a quite different situation for comparison, and the successfully descriptive formulæ, whether arrived at as empirical approximations or analytically, are totally dissimilar. We will consider first the evidence from *Malacosoma* larvæ.

III.

The experiments are very simple, and for that reason require considerable care. Each caterpillar, from a nest maintained on a branch of wild-cherry in the laboratory, is repeatedly caused to creep upon an inclined plane. In successive groups of "runs" of observations the inclination of the plane is altered. The surface is covered with coarse textured paper, affording a good foothold. The larva is started head downward, or with the axis horizontal, and its path is traced by gentle application of a soft pencil to the paper just behind the moving animal. The tests are made in a ventilated

dark room, under very weak non-directive red light. This is necessary, as during the periods of the day involved in the tests directive white light of low intensity suffices to overcome the geotropic effect; to this aspect of the matter we expect later to return. Suitable tests showed that the location of the observer did not influence the trails made; a possible influence of his breathing currents was excluded by an appropriate screen. Tests in which creeping was studied upon a tightly stretched cloth surface showed that although the character of the surface may influence the *speed* of progression it does not affect the angles of orientation. Nor did the pressure of the tracing point detectably modify the orientation. The tests were all made between the hours of 2 p.m. and midnight. The temperature in the dark room was between 21° and 24°C. Occasionally, the geotropism is reversed and becomes positive; this happens especially when the food supply is low. Erratic creeping is also sometimes encountered, but this is rare and can usually be traced to poor handling or to lack of food. The best trails are secured when creeping is at moderate speed, practically continuous, with the anterior end swinging not too vigorously nor too extensively from side to side. As with other forms, geotropic orientation is evident only during progression (this also holds for experiments upon a turntable). With increasing inclination of the plane, the speed of progression becomes greater, but at inclinations above $\alpha = 60°$ "hesitation" may be more pronounced, so that the net rate of progression appears to go through a maximum at about this inclination. In earlier experiments (Crozier and Stier, 1925–26) we avoided this effect of lateral head-movements by forcing the caterpillars to creep (vertically) upon a slender rod. We have used individuals 2.5 to 3.5 cm. long, as older individuals (approaching pupation) may tend to be erratic; moreover, it was desirable to reduce certain sources of variation, possibly connected with age, in view of the irreducible quantitative fluctuation of the geotropic sensitivity which we will shortly discuss.

Each animal was started first with one side, then with the other, downward. There is considerable evidence of fluctuating asymmetry in response. This sometimes takes the form of persistent turning to one side, so that for a period all the orientation angles may be to the left or to the right, no matter how the larva is started. More

commonly, the asymmetry is revealed in a slight but on the whole consistent difference, during a given set of trials, between the "right" and the "left" orientations. These differences have been discounted by the method of averaging the measurements from the individual trails. Persistent turning to one side has the advantage that the limiting value of the angle θ achieves more definite expression.

At inclinations of the creeping plane above 20°, sometimes above 15°, the caterpillar promptly orients upward, and usually strikes a quite definitely straight path. The angle (θ) of this path, on the plane (Fig. 1), in some instances changes from time to time, but in a

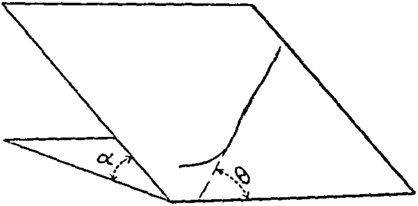

FIG. 1. Illustrating the definition of the angles and θ involved in the description of geotropic orientation of *Malacosoma* larvæ. The traced line of progression of the caterpillar, when creeping is started with the axis of the body horizontal, comes to make a definite angle θ on the inclined plane. In many instances the path is complicated, in ways discussed in the text.

manner which in the very great majority of cases is definite and abrupt. Illustrations are given in Fig. 2 (*cf.* Fig. 3). In all such cases each magnitude of θ was recorded. Change of direction was almost always associated with a brief pause in creeping, and with a more or less sharp alteration in the amplitude of the sideward bendings of the anterior end. To this point we shall later return. The change of direction may result either in an increase or in a decrease of θ; at higher values of α it is inappreciable. The theory of orientation expressed in earlier papers (Crozier and Pincus, 1926–27, *a*, *b*) leads to the expectation that within the area defined on the creeping plane by $+\theta$ and $-\theta$ the direction of movement is not constrained. Hence, "chance" further upward turning would be expected to produce false

high values of θ in at least a number of trails such as those fairly sampled in Fig. 2. An escape from this difficulty is, on the one hand, to secure a very large number of trails; and on the other to consider the results from those trails in which no obvious change of θ is apparent, and which in fact yield identical average magnitudes of θ. The total

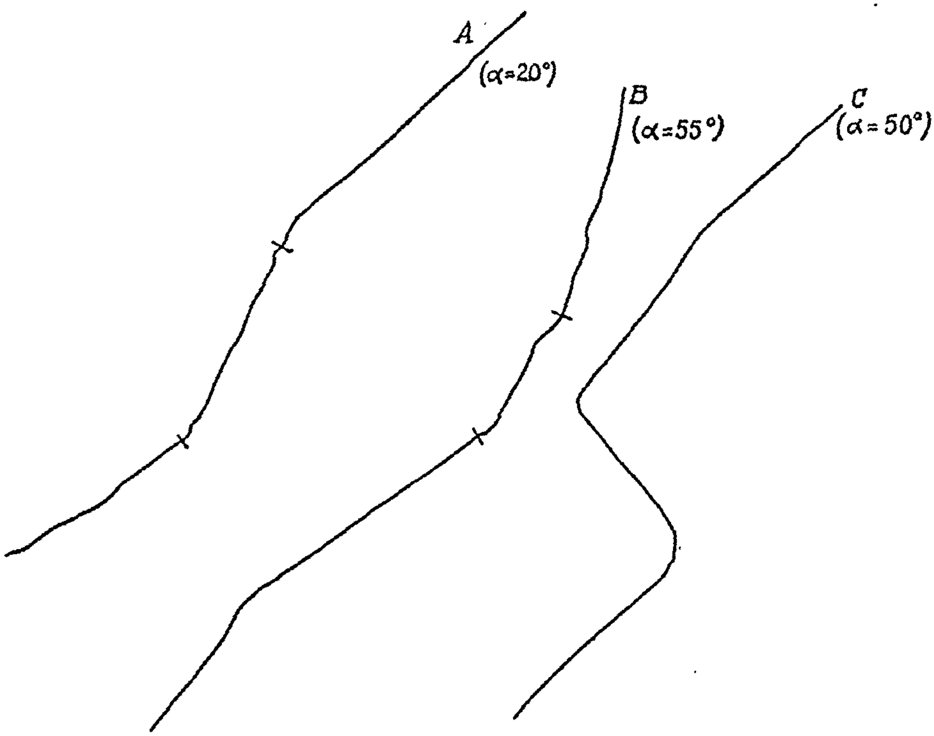

Fig. 2. Trails (× ½) chosen practically at random to illustrate abrupt changes of θ during progression on an inclined plane. (In each case the tilt of the plane, α, is given; the respective magnitudes of θ are only vaguely similar, in these instances, to the corresponding mean values.) The cross marks signify brief cessations of creeping. See text.

number of trails measured was 1,480. It is of course obvious that with a very rapidly creeping larva orientation may occur in such a way as to bring the axis of progression to a position beyond the limiting value of θ, in which case a widely curving trail may result, the final outcome being (given creeping over a sufficient distance), that the complimentary limiting position is ultimately assumed (*cf.* *C*, Fig. 3). The proportion of such trails encountered is quite small,

however. In any case the few curved paths are legitimately excluded from consideration, since our object is to deal with the nature of the limiting conditions for oriented progression.

Since the trails were usually taken upon sheets of paper 23 cm. wide (*i.e.*, high), it was possible that the handling required at the completion of a trail and at the starting of another might be of significance in connection with this variation. Experiments were

Fig. 3. To illustrate method of obtaining the angle of upward orientation (θ). The heavy lines are the paths of creeping as recorded by tracing the position of the posterior end of the animal ($\times \frac{1}{4}$). The light, dashed lines are the fitted slopes. Group *A*, caterpillar No. 15, $\alpha = 40°$; *B*, No. 18, $\alpha = 60°$; *C*, No. 10, $\alpha = 40°$. *C* shows a trail of the widely curving type referred to in the text.

therefore made upon a platform of 3 meters length, on which the spontaneous changes of amplitude of orientation, together with associated changes in the speed of progression, were equally visible in trails covering undisturbed creeping over the whole length of the platform. In fact, the mean amplitudes of orientation obtained in this way agree quantitatively with those obtained by the first method at the same inclinations. The relation between change of amplitude and alteration of the head movements was investigated by means of trails upon smoked paper.

IV.

The method of measurement of the angles of orientation is sufficiently illustrated by the examples in Fig. 3. The aim was to obtain a measure of the average angle of orientation (θ). Consequently

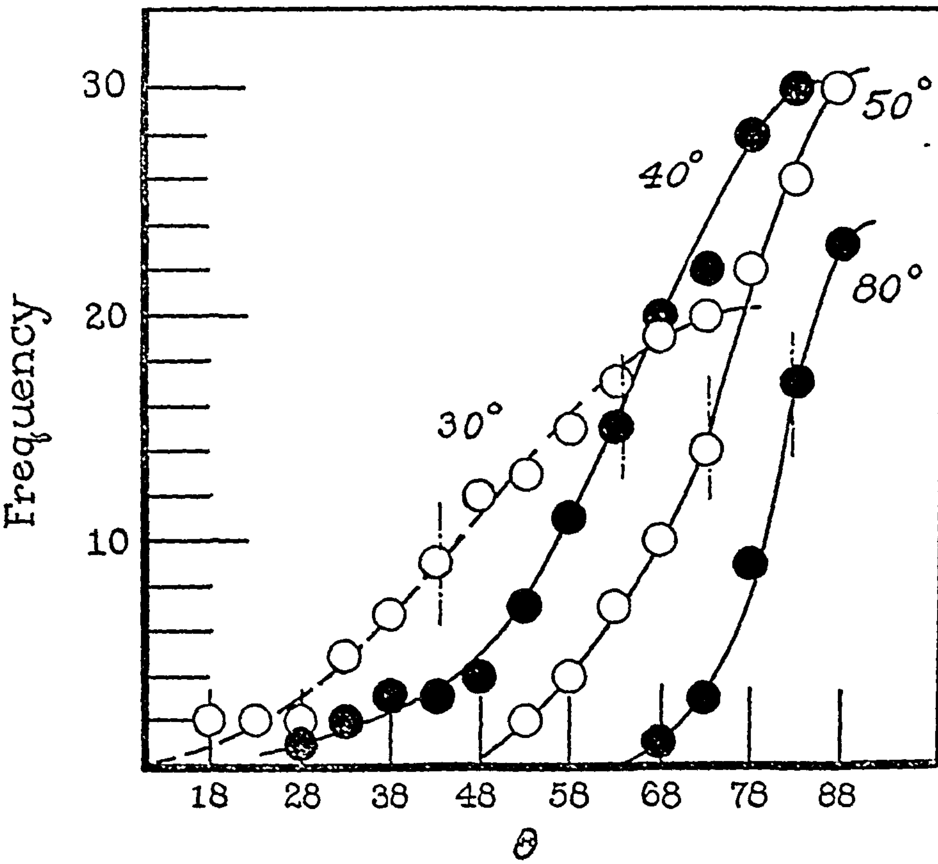

FIG. 4. Summated frequency distributions for readings of θ at α = 30°, 40°, 50°, 80°, with caterpillar No. 18. The means are located at the inflection points. The distributions are of a span decreasing with rise of α, and show slight negative skewness. The measurements here represented are typical for other individuals.

the 'right' and the 'left' trails for each individual, at a given value of α, were averaged separately from the 10 to 20 trails secured. The average θ was then taken as the mean of the 'right' and 'left' values, and these averaged for the different individuals. This procedure makes it difficult to give an adequate value of a P.E. for the

general average, except in terms of the contributing averages from the several individuals. Perhaps the most convenient method of illustrating the nature and the extent of variation in the data is to give plottings showing (1) dispersions of individual readings of θ, (2) the random (*i.e.*, fluctuating) character of the 'right'-'left' asymmetry, (3) dispersion of the means for the separate individuals. These points are illustrated in Figs. 4, 5, and 6.

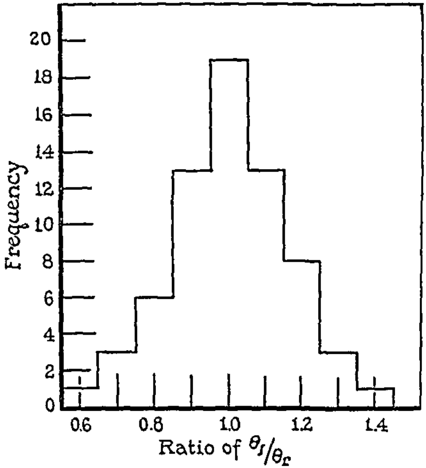

FIG. 5. Showing the random character of the asymmetry ratio θ_l/θ_r; data from 8 individuals, at various times, at several values of the inclination α. (The ratio for *left* and *right* orientations of any one individual may vary from 0.6 to 1.2 at different times.)

In comparing frequency distributions (Fig. 4) it is certainly illegitimate to construct histograms at all magnitudes of α with equal size classes for the observed θ's, because the recognizable behavior of θ (*cf.* Crozier and Pincus, 1926–27, *a;* Crozier and Oxnard, 1927–28, indicates plainly that the variability of θ (mean) is itself a function

of α. It is more convenient, for our present purpose, to provide a typical illustration of the conditions met with; in Fig. 4 this is done by giving dispersions of θ in the ogive form, from measurements with one individual. When data secured from all individuals used at one value of α are lumped together, the frequency plot may show pronounced negative skewness. This is due to the inhomogeneity of the group; the general mean now deviates from the average of the contributing means. The skewness is most marked at lower values of α,

TABLE I.

The extent of upward orientation (θ) in negatively geotropic larvæ of *Malacosoma americana* with various inclinations of the creeping surface (α). θ is given as the general mean of the averages of 10 to 20 trails with each of 6 to 22 individuals, each used at more than 3 (usually 6) values of α.

α	θ
15°	31.18° ± 0.59
20°	44.87° ± 1.87
25°	49.49° ± 2.46
30°	59.74° ± 2.04
35°	67.17° ± 9.72
40°	66.79° ± 1.50
45°	69.19° ± 1.95
48°	65.98° ± 2.00
50°	75.37° ± 0.49
55°	74.78° ± 1.86
60°	76.40° ± 1.15
63°	78.42° ± 2.25
70°	77.13° ± 1.45
80°	80.79° ± 0.82
85°	83.76° ± 0.82

and general theory predicts that the distribution should then be normal if the frequency classes are formed in a geometric series. The application of the theory of logarithmic frequency distributions to such cases will be discussed elsewhere. Speaking generally, it may be remarked that there is illustrated in this way merely a consequence of the fact that, to a sufficient approximation for most purposes, θ is a linear function of *log sin* α, and that variables contributing to erratic (low) values of θ,—that is, such as mask or compete with geotropism—are suppressed with an efficiency which is also a linear

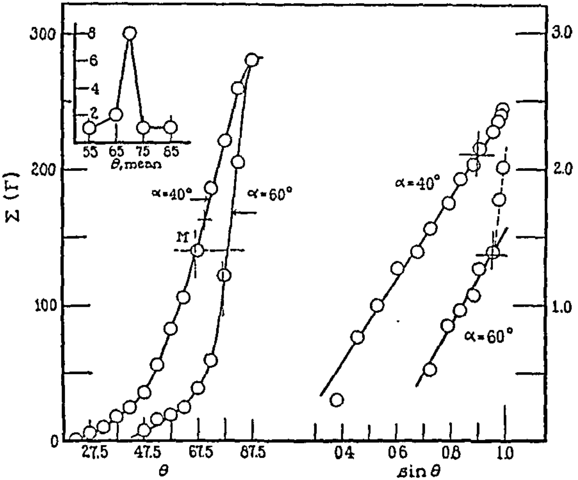

FIG. 6. Frequencies of θ as measured from trails of 13 caterpillars at $\alpha = 40°$ ($n = 280$). The curve shows some negative skewness, yet the group mean (M), the median for this distribution, and the mode are fairly close. The distribution of the means from the observations upon the separate individuals is given in the insert. The "tail," that is, the asymmetry due to excess low values of θ, should decrease with increase of α, and nearly in direct proportion to log sin α, if the general theory is sound. To test this precisely requires data of greater homogeneity than we now have, but the curve given for $\alpha = 60°$ ($n = 140$, plotted on a percentage scale) illustrates the relation qualitatively. The point may be examined in another way. As subsequently brought out, the product (sin θ) (sin α) should be a constant, statistically; therefore, with constant α the logarithm of the summed frequencies of θ should be found to be linearly related to *sin* θ, if attention be paid to those observed low values of θ which represent departures from the ideal magnitude due to some excitation the effects of which are not overcome by the geotropic excitation. The assumption here is that the orienting force is a function of sin θ (with α constant), and that negative departures from a mean value of θ, representing the "interference" of other kinds of stimulation, should be expected with a nearly logarithmic decrease as the magnitudes of the orienting stimulations associated with the departures increase. Graphs *C*, *D*, which are typical, show that this view is justified.

function of *log sin* α. This is of course the meaning of the fact, already abundantly demonstrated, that the variability of θ (for cases with normal frequency distribution; Crozier and Pincus, 1926–27, *a*, *b*, *c;* Pincus, 1926–27; Crozier and Oxnard, 1927–28) decreases in direct proportion to *log sin* α. At the highest values of α (*cf.* Fig. 4,

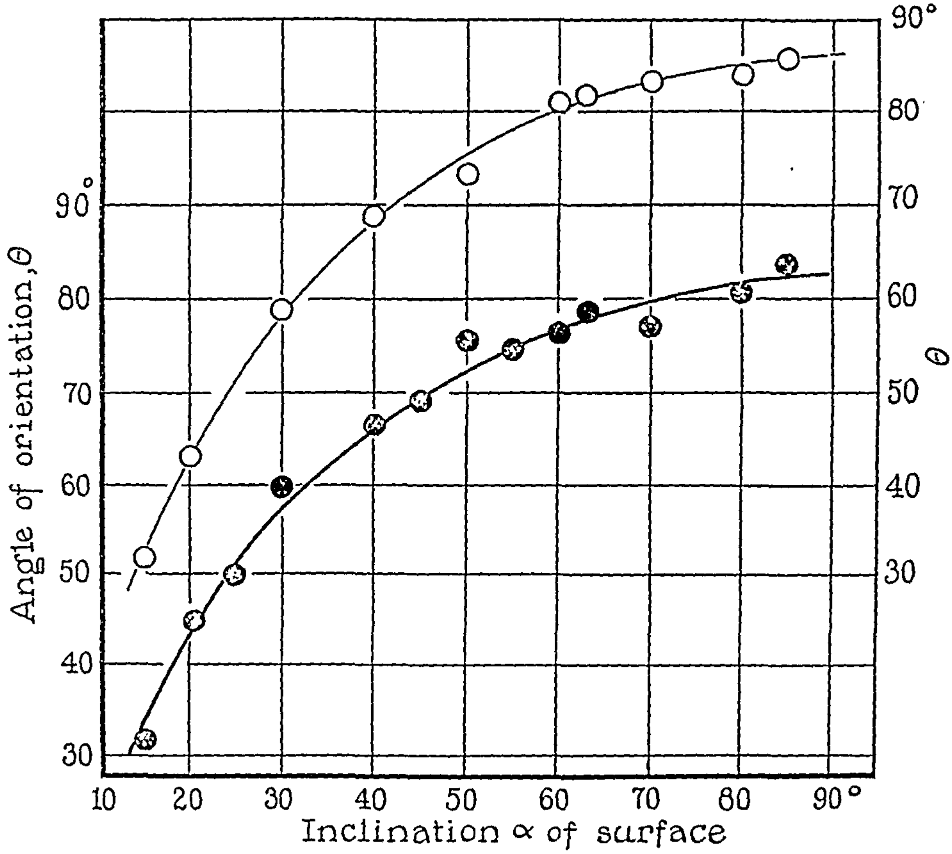

FIG. 7. Relation between tilt of angle of inclination (α) of plane from the horizontal and the mean angle of upward orientation (θ) on the plane (*Malacasoma* larvæ). Lower curve, means of the average θ's; upper curve, means from one individual. The curve is that found in Fig. 8.

e.g.) the distribution is slightly skewed, because θ cannot very well be $> 90°$.

The resulting mean angles of upward orientation obtained from experiments with 22 individuals are collected in Table I. The variability of the means decreases as α is made larger, but fails

to exhibit the regularity obtained in earlier measurements with young mammals (Crozier and Pincus, 1926–27, *a*, *b*, *c*; Pincus, 1926–27; Crozier and Oxnard, 1927–28). This is undoubtedly due to the fact that the mean values of θ from tests with different individuals have been averaged. Duplicate sets of experiments with the same larvæ

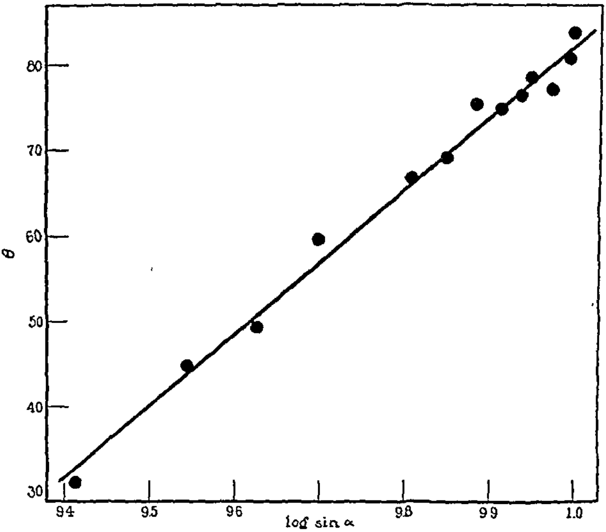

FIG. 8. Rectilinear relationship between mean θ and log sin α.

show excellent agreement. For example, at $\alpha = 40°$ two independent series of tests with caterpillar No. 1 gave $\theta = 56.4°, 58.5°$; at 20°, two series with No. 4 gave $\theta = 46.4°, 46.5°$; at 60°, two series with No. 8 gave $\theta = 72.5°, 72.4°$; at 80°, two series with No. 17 gave $\theta = 82.0°, 75.6°$.

A smooth relationship is apparent between the tilt (α) of the plane and the mean extent (θ) of the upward orientation. This holds as

well for the data from single individuals (*cf.* Fig. 7). As with rodents (Crozier and Pincus, 1926–27, *a*, *c*; Crozier and Oxnard, 1927–28) and slugs (Wolf, 1926–27), the angle θ is linearly proportional to *log sin* α, and hence to the logarithm of the component of gravity acting to produce downward pull on the plane. The relationship (Fig. 8) is precise enough to permit its use for experiments with compound fields of excitation, analagous to those already made with rats (Crozier and Pincus, 1926–27, *b*). But it is desirable to obtain another formulation, in terms of a structurally possible mechanism. This may easily be done by means of a simple assumption, which is not arbitrary because, for one thing, it is open to independent test.

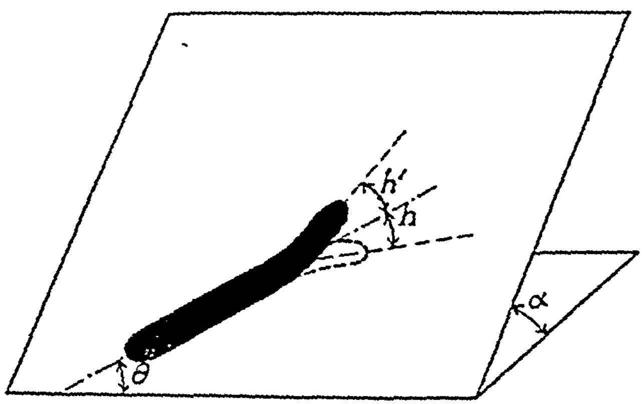

FIG. 9. Indicating definitions of α, θ, and the angles h, h', determined by the sideward excursions of the head.

With the terminology given in Fig. 9, let it be supposed that in steady progression, with a given amplitude of the sideward swings of the anterior end, the caterpillar turns upward until the difference between the tensions of the "head" upon the lateral musculature of the upper side in positions (1) and (2) is reduced to a constant (threshold) value. In applying this assumption to the average angles of orientation (Table I) it is assumed that the *mean* amplitude of the head swings, that is, the angle $2h$, is constant under all conditions of creeping, a view which mere inspection during the prolonged experimentation suffices to show cannot be very far wrong. The method of averages employed in getting the values of θ discounts

independent variations in muscular sensitivity on the two sides of the body, and the distribution of the "asymmetry" ratios shows that these, and h and h', are statistically equal.

On this assumption, from Fig. 9, at orientation

$$g\,[\cos(\theta - h) - \cos(\theta + h')] = const.,$$

where g is the effective component of gravity, $= K \sin \alpha$; then, directly,

$$(\sin\alpha)\,(\sin h)\,(\sin\theta) = K.$$

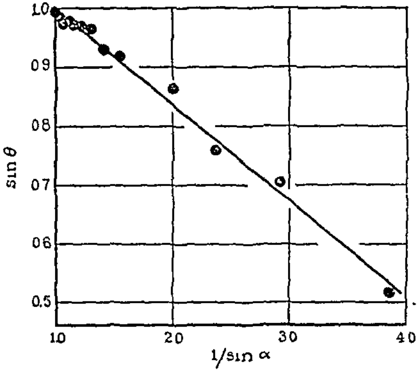

FIG. 10. Sin $\theta = -K/\sin\alpha + C$.

If h be constant,

$$\sin\theta \sin\alpha = K'.$$

If g be constant, θ must vary inversely with h. These two consequences we proceed to test. The first is shown by Fig. 10 to give excellent quantitative correspondence with the facts of observation.

The second consequence (which agrees with simple observation) can only be tested in a qualitative manner, but with a decisive result. By means of trails upon lightly smoked paper it is possible to measure, with fixed inclination of the plane (α), the relation between "spon-

taneous" changes of θ and the automatically recorded changes in the lateral swinging excursions of the anterior end. These changes are involved with spontaneous changes in the speed of progression, and thus contribute to the variability θ as seen in the "long trail" experiments already mentioned. The results of a number of such tests are collected in Table II. They show that when, with constant α, θ increases, the lateral excursions decrease in amplitude; hence the angle of the swings must decrease (since the structurally movable part is necessarily restricted to the head and thorax, hence it is not a question of mere posterior shifting of the origin of the swings). This relationship may be tested in another way. With a single

TABLE II.

Mean values of the width of the trail left on smoked paper by a *Malacosoma* larva at various angles of orientation (θ), creeping on a plane tilted at $\alpha = 20°$ or 40°.

θ	Width
	mm.
$30° \pm 3$	12.5
$45° \pm 4$	12
$60° \pm 3$	11
$75° \pm 2$	10.8
85°	10

individual it would be expected that, barring changes in "sensitivity," the "head angle" should decrease as α is increased, disregarding the magnitudes of θ. The "head angle" is again roughly measurable by the width of the path left upon the smoked paper by the mouth-parts and the cephalic "hairs" in creeping. A series of such tests gave the following values:

α	=	20°	50°	80°
Mean width of trail, *mm.*,	=	11	10	8.5

V.

The result regarding the interpretation of geotropic orientation in *Malacosoma* larvæ is thus held to be a close parallel for that in the case of the slug *Agriolimax* (Wolf, 1926–27), with the sole essential

differences (1) that the "head angle" is dynamically determined by the method of progression, rather than structurally fixed, and (2) that the downward pull appealed to as providing the stimulus for orientation is exerted upon the musculature of one side only, namely the upper. With an organism having a body so nearly cylindrical as the caterpillar's is, and supported along its length, except from the thorax forward, by means of the prolegs, there seems no alternative method whereby a source of differential excitation may be found such as is required to give the experimental results.

It has been pointed out (*cf. e.g.*, Parker, 1922) that in general the older mechanical theory of geotropic orientation meets with serious difficulties, at least when considered in the form that the weight of the body, and thus the axis of the organism, is disposed in a particular way in relation to the locomotor apparatus according to the action of gravitation upon the body as an inert mass. There are equally serious obstacles in the way of any general application of a "statolith" theory. Particularly, there is no apparent means, on such basis as provided by either of these views, whereby to account for the quantitative relationships between gravitational pull and the amplitude of orientation. There is left the appeal to the proprioceptive results of muscle tensions, already suggested to account for certain features of geotropism among insects (*cf.* Cole, 1917) and molluscs (Arey and Crozier, 1919; Crozier and Federighi, 1924–25; Cole 1926–28), although at that time without detailed support or development; and in certain cases among metazoans the possible directive effect by the pull of the weight of certain internal organs (*cf.* Crozier and Arey, 1919), as shown especially by Wolf in connection with the righting movements of the starfish (Wolf, 1925) and suggested by Parker (1922) for the orientation of the sea urchin *Centrechinus* (through the weight of the long spines; *cf.* also Bolin (1926)). Experiments by one of us (T. J. B. S.), now in progress, have shown that the deforming pressure of the pull of the body wall, in Holothurians, may be effective for some manifestations of geotropism, thus quite apart from the possibility of the pull of spines, or of internal organs (or of the statocyst function in synaptids (*cf.* Olmsted, 1917)).

The point of this digression is, that although the theory of statolith

function may be necessary for some forms, to account for the *direction* of geotropic orientation—upward or downward—and perhaps in dealing with certain forms of experimental reversal of the orientation, the deforming pressures of the body weight upon the parietal musculature, or upon the muscles of appendages, must be taken to provide the bilaterally differential effect necessary to govern the occurrence and the *amount* of orientation as a function of the intensity of the exciting force; the effect of added loads is definite confirmation (Crozier and Pincus, 1926–27, *a;* Pincus, 1926–27). It is this aspect of the matter which is the most inviting. Not only does it permit precise description of behavior in terms of simple mechanisms, but it seems to give a fairly direct approach to the investigation of the physiology of muscle receptors.

VI.

SUMMARY.

The geotropic orientation of caterpillars of *Malacosoma americana* during progression upon a surface inclined at angle α to the horizontal is such that the path makes an average angle θ upward on the plane, of a magnitude proportional to *log sin* α. More precisely, the product $(\sin \alpha)$ $(\sin \theta)$ is constant. This is traced to the fluctuation of the pull of the head region upon the lateral musculature of the upper side during the side to side swinging implicated in progression.

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THE INFLUENCE OF pH UPON THE CONCENTRATION POTENTIALS ACROSS THE SKIN OF THE FROG.

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INTRODUCTION.

In 1890 Ostwald (1) suggested that the electromotive phenomena in living tissues might arise because the living membranes, in common with many non-living structures, prevent or retard the passage of the ions of one electrical sign while permitting the movement of ions of the opposite sign. As a result of this selective behavior electrical stresses may arise in any situation where there is an ionic concentration gradient across a membrane and these stresses will become manifest in potential differences which can be measured by appropriate devices. While the application of this conception has proven valuable in several fields of investigation the attention of workers has in the main been focused upon the differential movement of ions through membranes, rather than upon the equally fundamental inquiry as to why membranes behave in this manner. The present communication will deal with certain observations which are believed to bear upon this latter problem.

These studies were suggested by the observations of Matsuo (2), Mond (3), and Fujita (4) that across protein membranes the concentration P.D.'s are greatly influenced by pH; that above the isoelectric point of the protein the more dilute solution is electropositive, whereas below it the polarity is reversed. We were further moved to attempt these experiments by the report of Mudd (5) that mammalian serous membranes show an electroendosmotic reversal point in a pH range (4.3–5.3) which strongly suggests that this phenomenon is controlled by a protein constituent of the membrane.

Such studies suggested to us that if, in intact animal membranes,

a similar correlation between the electromotive phenomena and pH could be demonstrated we might secure a deeper insight into the mechanism of bioelectric effects, and might be better able to decide between the views of Beutner (6) and of Höber (7) and his coworkers (2, 3) as to the particular chemical substance which is responsible for the production of P.D. in living tissues. The latter group of workers has recently contended that protein models simulate the electromotive behavior of living tissues more closely than do Beutner's oil-water systems.

We have investigated the relationship between pH and electromotive phenomena in a number of animal membranes and have so far discovered striking reversal effects in at least four different materials. The present report will deal with experiments carried out on the skin of the frog, in which the results have been particularly clear. We have been unable to discover in the literature any previous detailed study of the influence of pH upon the electrical behavior of this membrane. Uhlenbruck (8) has seen the electromotive reversals which we will discuss, but he mentions them only incidentally and does not, we believe, appreciate their significance. In a recent paper which came to our attention after the completion of our own work, Rein (9) has reported observations on another material which are in general agreement with our own results. He finds that the concentration P.D. which he can demonstrate in the human skin is reversed when the electrical charge upon it is made positive by the H ion or by Al^{+++}. These papers appear to constitute the only studies of the sort which have been carried out on animal membranes.

References to the older literature on the electromotive phenomena of the frog's skin are given by Hashida (10) and Uhlenbruck (8). Wertheimer (11) has published extensive studies on the permeability of this membrane.

When the living skin of the frog is removed from the body and so mounted that its two surfaces are bathed by an isotonic Ringer's solution, a difference of potential may be demonstrated across it, the inner surface being electropositive. Upon stimulation of the cutaneous nerves characteristic changes in this skin potential may be obtained, presumably associated with the activity of the skin glands. These intrinsic electrical effects are known to depend upon the pres-

ence, in the solutions, of Na ion, which may be replaced, in part at least, only by Li (8, 10).

Another category of electromotive phenomena is observed when salt solutions of different concentrations are applied to the two surfaces of the skin. Concentration P.D.'s of quite a high order, up to half of the theoretical maximum, are then obtained. In a membrane in which the normal skin potential persists, the actual P.D. measured in the course of such a concentration study is the algebraic sum of the normal skin potential and the concentration potential.

The present study concerns itself entirely with concentration P.D. measurements under conditions where the skin potential has been abolished. When this occurs the skin is presumably no longer alive. We have reason to believe that in this material such electromotive reversals as we shall discuss are not possible in life. Thus we find that when Ringer's or NaCl solutions are employed the skin potential diminishes as the solutions are made more acid and disappears at almost exactly the same pH (3.8–4.2) as that at which the reversal of concentration potential occurs. When the solutions are made more alkaline again the skin potential does not reappear; the concentration potential, on the other hand, assumes its original polarity and finally reaches the same value as that previously observed when skin potential was present. The ability of the skin to develop concentration potentials is obviously not dependent upon the presence of a skin potential. It depends rather upon the presence of a physical structure which may persist for many hours after the membrane has ceased to function as a living tissue. A study of such a membrane may, we believe, give us valuable information concerning the electrical behavior of that structure in life.

The frog's skin has, of course, a somewhat complex structure, consisting of at least two distinct layers. The outer epithelial layer, containing many glands, is almost exclusively responsible for the electromotive phenomena. When this outer layer has been removed the inner portion, composed of fibrous tissue and smooth muscle, and containing nerves and blood vessels, is unable to develop concentration P.D.'s of any magnitude.

Preliminary reports (12, 13) of this work have been published and may be consulted for a brief summary of the results which have been obtained upon other materials.

Method.

Fig. 1 presents, in diagrammatic form, the experimental arrangements used for the determination of P.D.'s, except in certain special cases later to be described in which some modification was necessary. In general the experiments follow a familiar procedure, and offer no exceptional features. The membrane, consisting

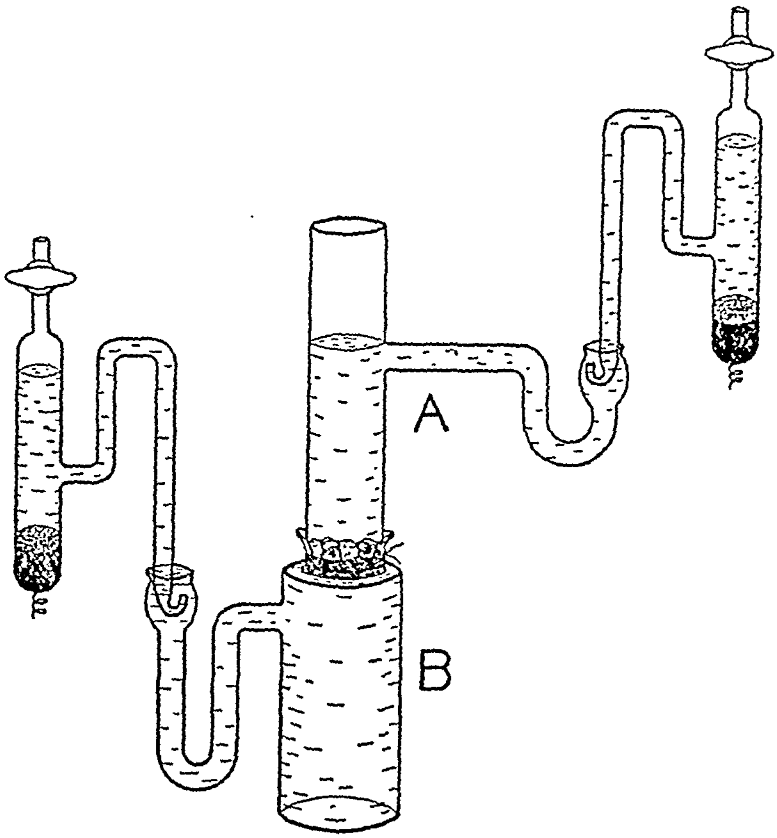

FIG. 1. Diagram of apparatus. The skin, mounted over the end of vessel *A*, is brought in contact with the salt solutions in vessels *A* and *B*. Electrical connections to the potentiometer are made through saturated KCl calomel half-cells.

of a piece of skin cut from the back, is mounted on the lower end of a glass cylinder, *A*, the inside of the skin being turned toward the inside of the chamber. One solution fills the chamber and extends through the side tube to the smaller vessel at its end. Electrical connection to one pole of the potentiometer is made through a saturated KCl calomel half-cell. The other solution is placed in a glass vessel, *B*, with a similar side tube. Electrical connection to the other pole of the potentiometer is made through a second calomel half-cell. The instrument used is a

Leeds and Northrup portable hydrogen ion potentiometer. A capillary electrometer is used as the null instrument.

RESULTS.

The phenomena now to be described may be studied in a variety of ways. We have taken at least three distinct steps in the analysis of the problem, beginning with a roughly qualitative demonstration, proceeding through a more careful study with unbuffered solutions, and ending in a series of determinations with acetate buffers, in which the pH is more rigidly controlled, and in which the electromotive effects are shown to be closely related to the electrical charge of the skin. These three portions of the study all contribute to the argument and will be described in order.

1. When solutions of .1 N KCl, made up in the usual manner, and at a pH above 5.0, are brought in contact with the two surfaces of the skin, it is observed that the skin potential originally present diminishes rapidly and finally disappears after a few minutes. The P.D. of the system thereupon becomes zero. If now the solution in vessel *B* is replaced with .001 N KCl and the system again examined, a concentration potential is observed, the voltage often reaching 60 millivolts. The more dilute solution is electropositive. This P.D. is a membrane potential in the strict sense. It is very much greater than any diffusion potential which could possibly arise between the two solutions. The magnitude of the diffusion potential between .1 N and .001 N KCl may be calculated, and comes out to be .8 millivolts only; the polarity is the reverse of that actually observed when the membrane is present. The membrane itself must obviously be in some way responsible for the P.D. produced.

A sudden change in pH may now be produced by adding a few drops of .1 N HCl to the dilute solution. When, by such an addition the pH is lowered to 3.0 or thereabouts a striking change is observed in the concentration potential. The P.D. previously present rapidly diminishes to zero, to be succeeded by a P.D. in the opposite direction which builds up to a maximum which is usually not greatly different from the value originally found in the other direction before the addition of the acid, although in general the terminal values tend to be less.

The ionic concentrations in vessel *B* are of course affected by the addition of acid. Thus at pH = 3.0 the Cl ion concentration is no longer .001 N in the dilute solution but .002 N, nearly. An ionic concentration gradient still exists, therefore, in the same direction as before, and the P.D. observed after acid treatment is a concentration potential whose polarity is determined by some change within the membrane induced by the H ion.

The sudden reversal in P.D. may be observed by setting the potentiometer to zero and allowing the full force of the unbalanced membrane potential to deflect the meniscus of the electrometer to one side of its zero position. Upon the addition of the acid the meniscus is seen to move rapidly through the zero position to the other side, indicating the electromotive reversal.

By successive adjustments of the pH, now to the alkaline and now to the acid side, the P.D. may be reversed many times at will. The pH of the internal solution has little or no effect upon the behavior. It may be lowered and raised simultaneously with that in the external solution, or it may be left more alkaline throughout; in each case the electromotive reversals are practically identical. Very definitely the phenomena are connected with the epithelial layer of the skin which is in contact with the more dilute solution.

2. More complete information as to the form of the reversal curve and as to the reversal point is obtained when the pH is shifted more gradually by the application of a series of solutions at different pH's. By the addition of HCl the pH of a .1 N KCl, and of .001 N KCl series is adjusted to cover the range from 3.0 to 5.6 in steps of .2 pH. As has been pointed out, the ionic concentration difference between concentrated and dilute solutions is not constant in such a series, but even at pH = 3.0 the ionic ratios are still 50 to 1, nearly, and the gradient is always in the same direction over the whole range. When the concentration P.D.'s across the skin are measured by the application of such a series of solutions, the pH being the same on both sides of the membrane at each reading, typical reversal curves such as those shown in Fig. 2 are secured. The reversal points vary considerably from skin to skin, and are influenced to some extent by the direction from which the reversal point is approached. This is almost certainly due to a failure to secure a rigorous control of pH within the membrane

by the application of such an unbuffered series. This effect disappears in the determinations with acetate buffers, later to be described. The form of the curves obtained with KCl solutions and the reversal points indicated must therefore be accepted as approximate only, but as giving a correct picture of the general nature of the phenomena, and as demonstrating that the effects cannot possibly be due to diffusion

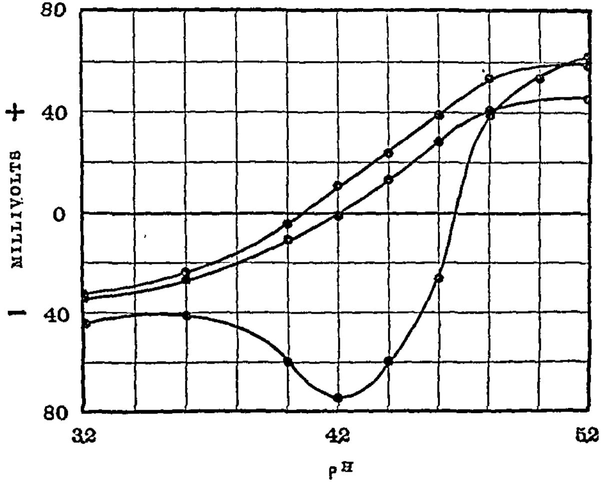

FIG. 2. Concentration P.D.'s between .1 N and .001 N KCl, as influenced by pH. The three curves represent the electromotive behavior of three different skins. The sign is that of the dilute solution.

potentials, or to any other factors involving the solutions only, but must be referred to changes in the membrane achieved through the influence of a varying pH.

The magnitude of the voltage observed at any pH is influenced by the manner in which the dilute solutions are applied. Throughout all determinations it is the custom to wash the outer surface of the skin through three changes of the dilute solution and to stir that

solution during the whole time in which the electromotive readings are being secured. If such precautions are not taken the P.D.'s, while definite, are considerably lower in value than when the dilute solution is continually renewed against the membrane. This effect is probably due to diffusion of salt through the membrane from the more concentrated solution, with a consequent reduction in the magnitude of the ionic gradient immediately across the membrane, and an associated diminution of electromotive force.

During the period in which the outer surface of the membrane, newly removed from contact with .1 N KCl, is adjusting itself to the dilute solution, many curious transient electromotive effects have been observed which we are at present quite unable to interpret. These effects were much less marked in the experiments with buffered solutions, described below. Immediately after the application of dilute KCl there may appear a large P.D. in a direction opposite to that which is found later after the system has come more nearly into a new equilibrium. This P.D. rapidly vanishes, to be replaced, within a minute or 2, by a P.D. in the opposite direction which builds up to a fairly constant maximum after 5 minutes. The readings here reported are in all cases these terminal values.

3. The final and most carefully controlled experiments have been carried out in Na acetate-acetic acid buffers. Preliminary tests demonstrated that a satisfactory concentration P.D. can be developed across frog skin when two different concentrations of these buffers are applied to the two surfaces. Inasmuch as over the whole effective range of this buffer (pH 3.6 to 5.8) the dissociation of the acetic acid is very slight and since we have every reason to believe that the undissociated molecule can make no contribution to the production of electromotive effects of the sort under discussion the concentration P.D. observed must be referred almost entirely to the acetate.

In the first experiments a series of buffers, .2 M in strength with respect to acetate, were employed as the more concentrated solutions. When such a solution at pH = 5.8 is applied to both surfaces of the skin, the skin potential initially observed rapidly disappears and the P.D. of the system becomes zero. If, now, the solution in contact with the outside of the skin is replaced by a tenfold dilution of the stock solution, a P.D. appears, the dilute solution being electropositive.

Similar readings may now be repeated in solutions becoming progressively more acid, and reversal curves similar to those already described are secured. These curves, however, are very far from being symmetrical with respect to the line of zero potential, the voltages on the acid side of the reversal point reaching much higher values than those obtained on the alkaline side.

It soon became evident that it is not sufficient to maintain a constant ion ratio, as the pH is varied, but that the total ion concentration must also be controlled. It has long been recognized that the magnitude of electrokinetic effects and allied phenomena are markedly influenced by the ionic concentration, being maximal over a certain dilute range of solutions, diminishing in more concentrated solutions, and finally disappearing entirely at or near moleculor values. It seemed reasonable to suppose that some similar effect was involved in the present study. Accordingly a new series of buffers was made up, by appropriate dilution of the stock solutions, having in every case a Na acetate concentration equal to that of the stock solution at pH 4.0. In this solution that concentration is .036 M. This series is used for the more concentrated solutions. A second series of dilute solutions is made up by a twentyfold dilution of the first series. An ionic gradient is therefore secured in which, over the whole range of the determinations, total ion concentration as well as ion ratio is very *nearly constant.*

Such dilutions cause a shift in the pH toward the alkaline side, but even when the original stock solutions are diluted a hundredfold the resulting pH change is not more than .2 of a pH unit. As a result of this shift the dilute solution is always slightly more alkaline than the concentrated solution, but in both the H ion concentration is negligible as compared with the salt, and no effect on the direction or magnitude of the concentration potential is produced, except in so far as the changing pH modifies the character of the membrane itself. In every case the actual pH values in the solutions, after both the first and the second dilution, are determined by the quinhydrone electrode. Contact with the tissue sometimes produces a slight change in the pH of the applied solutions. The determination of pH in the more dilute solution, therefore, is carried out on that sample last applied to the membrane, immediately after the electromotive reading has been

taken. The pH values here given for the electromotive measurements are always those thus determined, since we know that it is the pH of this dilute solution which controls the electromotive reversals.

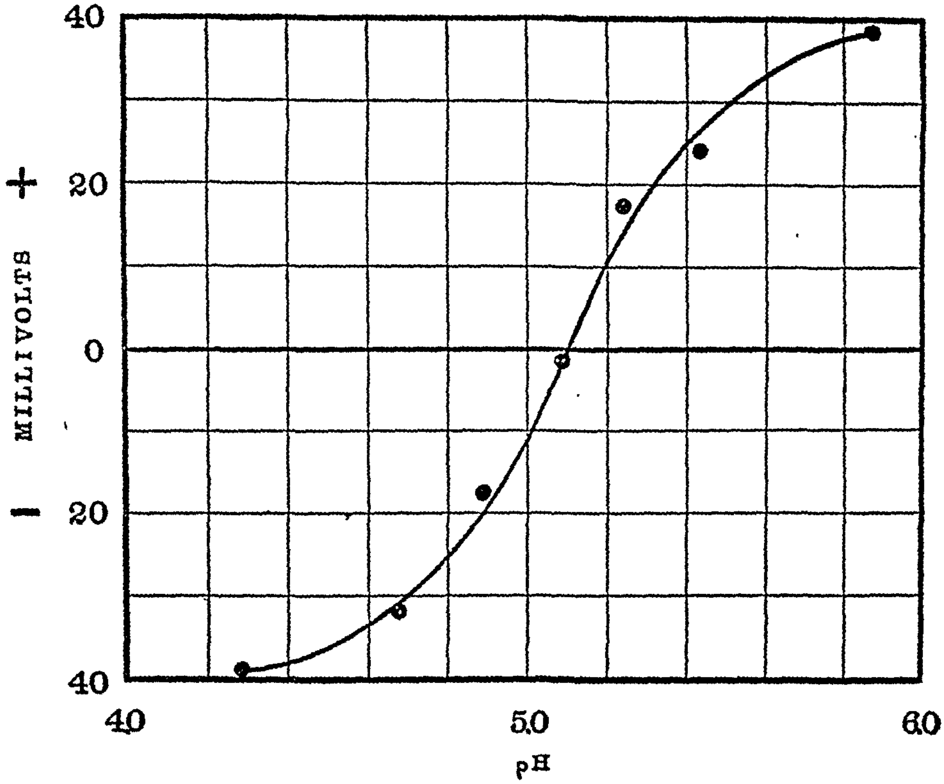

FIG. 3. Concentration P.D.'s between 0.036 N and 0.0018 N Na acetate, as influenced by pH. The sign is that of the dilute solution. The readings are as follows:

pH	P.D.
5.88	+38.8 mv.
5.43	+23.9 "
5.24	+17.0 "
5.09	− 1.4 "
4.89	−17.7 "
4.68	−31.6 "
4.29	−39.0 "

When the ionic concentrations are kept constant in the manner just discussed the reversal curves assume a more symmetrical form. A very good example of such a curve is shown in Fig. 3. At every point the concentration gradient is .036–.0018 M Na acetate. The

reversal or "isoelectric" point comes at pH = 5.1. Above this point the dilute solution is electropositive; below it this solution is electronegative.

These electromotive reversals are closely correlated with the membrane charge. It has been found possible, in a group of experiments, to secure an electroendosmotic determination of this charge. In these experiments the method and apparatus described by Mudd (14) have been used. The determination is not easy to make since the movement of water through the membrane tends to disintegrate it. Particularly is there a tendency for the outer epithelial layer to become separated from the inner layer of the skin, and in experiments on electroendosmosis we have never been successful in approaching the reversal point from the acid side without having this separation appear. All trustworthy determinations have been secured by approaching the reversal point from the alkaline side.

This difficulty appears to arise mainly from the fact that the two layers of the skin are very different in their electrical characteristics. It has previously been stated that the electromotive forces observed across the whole skin are almost entirely produced by the epithelial layer. It is also very apparent that the electroendosmotic transport of water through the whole skin is quite small in amount, compared with that which occurs when the epithelial layer has been removed. It follows, as a result of this difference, that, as long as the two membranes *remain bound together in the normal way, the epithelial layer* dominates the electroendosmosis, even as it does the electromotive phenomena. When the two membranes are separated, and particularly when actual openings appear in the epithelium, the amount of water which passes is determined almost entirely by the inner membrane. As a result the epithelial layer, if still intact, may be raised up in the form of a blister. A reversal in the direction of the current brings about a removal of this collection of fluid. In both cases the readings actually secured represent only the amount of fluid which has passed the inner membrane. Curiously enough the pH reversal point for this inner membrane is very different from that for the epithelium, and does not lie within the whole range of the acetate buffers, but at some higher pH value. A few determinations with Sørensen's phosphates have indicated an electroendosmotic reversal point at about 6.2–6.4.

When the two membranes have separated, therefore, no correlation can be established between the electroendosmotic and the electromotive behavior. But when they remain attached, so that the epithelium controls both phenomena, a close correlation is discerned. Table I gives the pH reversal points for both as determined in three experiments in which electroendosmosis was studied alone, eight experiments

TABLE I.

Reversal Points for Electroendosmosis and Potential Difference.

Experiment	Electroendosmosis	Potential difference
	pH	*pH*
1		5.09
2		5.12
3	5.19	
4		5.09
5	4.94	
6	5.24	5.20
7	5.19	5.15
8		5.08
9		5.20
10		4.97
11	5.03	5.10
12	5.07	4.96
13	5.23	
14	5.08	4.93
15	5.09	5.04
16		5.18
17	5.21	5.00
18		5.07
Average	5.12	5.07

in which the electromotive forces were measured alone, and seven experiments in which both were measured simultaneously.

For this last group of experiments a modification of the above described technique was used. It was found possible, after securing an electroendosmotic determination in the more concentrated solution at each pH (Na acetate always = .036 M) to read the concentration P.D. at that pH without removing the membrane from the electroendosmosis apparatus. This was done by bringing the more dilute solution (Na acetate = .0018 M) into contact with the epithelium and making electrical contact to the potentiometer through two saturated

KCl calomel half-cells, one dipping into the dilute solution, the other making connection with the inside more concentrated solution through a side tube on the electroendosmosis apparatus. After the attainment of approximate constancy in the electromotive reading the final value was accepted as giving the concentration P.D. at this pH. The

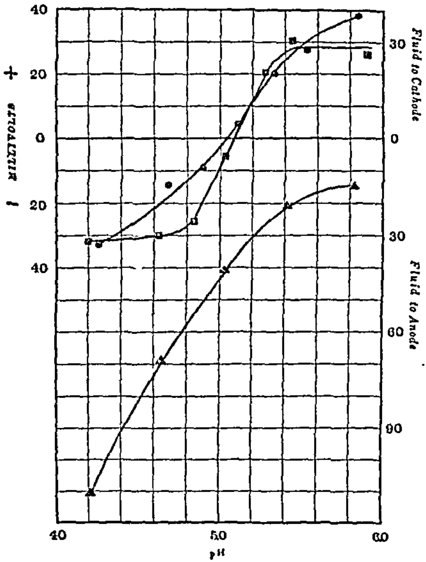

FIG. 4. Correlation between concentration P.D.'s and electroendosmosis. Ordinates at right represent the c. mm. of fluid transported in 15 minutes by a current strength of 6 ma.

● = P.D. in whole skin.

□ = Electroendosmosis through whole skin.

▲ = Electroendosmosis through skin after removal of epithelium.

solutions were then both replaced by the concentrated solution at the next lower pH and again an electroendosmotic and an electromotive reading were taken in succession at the new pH.

The electromotive reversal points are also in a pH range characteristic for animal proteins. These similarities indicate that in this material at least proteins are responsible for the electrical effects, and lend support to the views of Höber (7) and his coworkers.

In some of the systems which Beutner (6) has described electromotive reversals have been found which at first glance might be thought to be similar to, or identical with, the effects which we have discussed. Beutner has found that when such a substance as salicylaldehyde is used for the "oil" or water-immiscible phase in his models, the more dilute solution, in a KCl concentration series, is electropositive. When a basic substance, such as toluidine, is used the polarity of the concentration effect with KCl is in the opposite direction, the more dilute solution being electronegative. In more complicated systems in which the water-immiscible phase contains both an acid and a basic component the polarity is in one direction when an HCl concentration series is applied, and in the other direction when NaOH is used. Such effects are not, we believe, identical with the phenomena here described. In the first place it has not been shown that such electromotive reversals as Beutner secures are sharply defined within a narrow pH zone. Furthermore it comes out in his experiments that when the water-immiscible phase is acid the dilute solution is electropositive, when alkaline it is electronegative. This is the reverse of our own findings in which the dilute solution is electropositive on the alkaline side of an isoelectric point, electronegative on the acid side.

Certain it is that neither lipoids, nor proteins, nor any other particular chemical substances are necessary for the development of concentration P.D.'s which may simulate bioelectric effects. The glass electrode of Haber and Klemensiewicz (16) and the paraffin, wax, and collodion membranes of Michaelis and his coworkers (17–19) across which concentration P.D.'s of a high order may be demonstrated, are sufficient to show that we are dealing with some very general phenomenon. The mere imitation of bioelectric effects by models is therefore not sufficient to elucidate the specific mechanisms present in the living membrane. When it is found that animal membranes act as do protein membranes in respect to the influence of pH upon their electromotive behavior, we are justified in inferring that proteins are responsible in a specific way for bioelectric effects.

We have been led by the correlations above described and by many other similar observations on other materials to the view that there is some fundamental relation between the electrical charge present upon the surfaces and pore walls of an organized membrane and the ability of ions to penetrate the structure. In the frog skin it is clear that when the charge on a membrane is negative, above an isoelectric point, the penetration of cations is more easy than that of anions. This is true if we accept the polarity of the concentration effect as a trustworthy criterion for the determination of which ion penetrates more readily. Michaelis and his coworkers (20, 21) have secured very satisfactory evidence that this view is correct. Above the isoelectric point, therefore, the membrane appears to retard the movement of those ions which are of the same electrical sign as itself, and to permit the movement of ions of the opposite electrical sign. This differential effect disappears when the membrane charge has been reduced to zero. At more acid values the relationships are reversed; the polarity of the concentration effect now indicates that negatively charged anions pass more readily through a positively charged membrane than do positively charged cations. We are led to the view that electrostatic forces between charged surfaces and ions in the solution immediately in contact with those surfaces are responsible for these effects, the membrane charge operating to retard or prevent the entrance of ions of the same sign into the narrow pores through the membrane which are believed to constitute the pathways for ionic movements.

A different interpretation has been given by Bethe and Toropoff (22) and accepted by Michaelis (23) and Rein (9). According to this view the retardation of the ion of one sign is due to its selective adsorption upon the pore walls of a membrane, its companion ion being still free to move. Membrane charge, determined by such an adsorption, is thus a result rather than a cause of the ionic immobilization. We are unable to harmonize this conception with our own findings. In a membrane whose electrical behavior is determined by an amphoteric component the membrane charge is sufficiently explained in terms of the dissociation state of the ampholyte; the selective adsorption of ions from the solution need not be invoked. Above the isoelectric point, in such membranes, anions appear to be retarded, and, accord-

ing to the view of Bethe and Toropoff, this must occur because anions are selectively adsorbed. We must therefore imagine the adsorption of negatively charged anions upon surfaces which are already negatively charged by virtue of their own intrinsic ionogenic tendency. Such a situation seems to us to be without precedent, and we are led to suggest the alternative view which has been outlined. Even in other membranes in which membrane charge unquestionably arises from selective ionic adsorptions we feel that this charge, once developed, may operate to retard or prevent the movement, into the membrane, of other ions of the same sign still free in the solutions.

It will have been recognized that this conception as to the mechanism of bioelectric effects is not original with us. Similar views have been suggested by Girard (24–26), Haynes (27), Loeb (28), Risse (29) and others. Bayliss (30) and Jacobs (31) have recognized the possible intervention of such a factor in the control of permeability. The conception can readily be associated with the observations of many workers (32–34) that electrically neutral molecules penetrate living membranes more readily than do ions.

It may be remarked, in conclusion, that the electrical charge of a membrane is certainly not the only physical factor involved in the determination of electromotive effects or of permeability relations. The size and number of the pores, the thickness of the membrane, and other physical characteristics are of great importance. In many very porous membranes the P.D.'s developed are not membrane potentials in the strict sense, but diffusion potentials in large part, somewhat modified by the membrane, as Loeb (28) has pointed out. In those fairly porous membranes which Loeb, and Bartell (35) and his collaborators have used for studies of anomalous osmosis, membrane charge appears to be of slight influence in determining the P.D. across the membrane. The correlation which we have discussed becomes evident only in less permeable membranes in which, we believe, the dimensions of the channels through the membrane are so far reduced that electrostatic effects between the charged surfaces and ions in the solution dominate the electromotive behavior.

SUMMARY.

The production of concentration P.D.'s across the skin of the frog is very intimately related to the pH of the applied solutions. On the alkaline side of an isoelectric point the dilute solution is electropositive; on the acid side this solution becomes electronegative.

When the pH is suddenly lowered from a value more alkaline than this isoelectric point to one considerably more acid the change in polarity may occur within a few seconds. The effect is reversible.

When a series of unbuffered solutions at different pH values are applied reversal curves may be obtained. When the concentration gradient is .1 N–.001 N KCl the reversal points lie between pH 4.1 and 4.8.

When studied in acetate buffers this electromotive reversal is found to be closely correlated with the electrical charge upon the membrane, as determined by electroendosmosis through it. Reversal occurs between pH 4.9 and 5.2.

It is concluded that the electromotive behavior of this material is controlled by some ampholyte, or group of ampholytes, within the membrane. This ampholyte is probably a protein.

On both sides of their isoelectric point these membranes, in common with protein membranes, behave as if they retarded or prevented the movement through them of ions of the same electrical sign as they themselves bear, while permitting the movement of ions of the opposite sign. It is suggested that this correlation arises because of electrostatic effects between the charged surfaces and ions in the solution.

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